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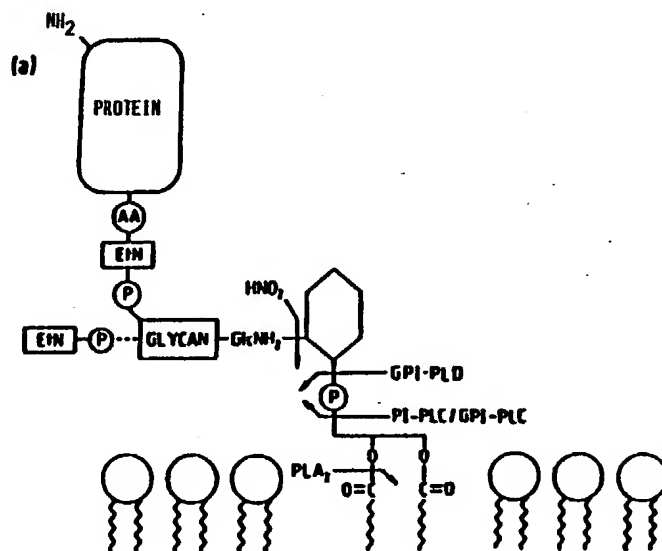
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(54) Title: USE OF p97 AND IRON BINDING PROTEINS AS DIAGNOSTIC AND THERAPEUTIC AGENTS



(57) Abstract

The invention relates to a GPI-anchored p97 and a soluble form of p97 and derivatives thereof and methods for preparing the same. Methods of using p97 in modulating iron transport, in the delivery of therapeutic agents, and in the treatment of conditions involving disturbances in iron metabolism are described. The treatment and diagnosis of Alzheimer's Disease in view of the finding that p97 and transferrin receptor are markers for microglial cells associated with senile plaques are also described.

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USE OF p97 AND IRON BINDING PROTEINS AS  
DIAGNOSTIC AND THERAPEUTIC AGENTS

FIELD OF THE INVENTION

TECHNICAL FIELD

5       The present invention relates to GPI-anchored p97, a  
secreted form of p97 and derivatives thereof; methods of  
using p97 in modulating iron transport, in the delivery of  
drugs, and in the treatment of conditions involving  
disturbances in iron metabolism; and methods of treating  
10   and diagnosing Alzheimer's Disease.

BACKGROUND OF THE INVENTION

Iron is a fundamental component required by all cells  
for growth and normal physiological processes (Crichton,  
R.R. and Charloteaux-Wauters, M. Eur. J. Biochem. 164:485-  
15   506 and Ponka, P. et al, Iron Transport and Storage, CRC  
Press, Boca Raton, Ann Arbor and Boston, 1990). Rapidly  
proliferating cells have a higher iron requirement than  
quiescent cells. In humans this iron requirement is  
thought to be provided by the binding of iron to the major  
20   serum iron-transporting protein, transferrin. Transferrin  
bound to iron can bind as a complex to the transferrin  
receptor expressed on the plasma membrane (Ponka, P. et  
al, Iron Transport and Storage, CRC Press, Boca Raton, Ann  
Arbor and Boston, 1990). After binding, the  
25   iron/transferrin/transferrin receptor complex remains  
membrane bound and is concentrated and then endocytosed  
via endocytotic vesicles. The endosomes become acidified  
and the iron is released from the complex within the cell  
and the apotransferrin remains bound to the receptor and  
30   is recycled to the surface where it is released to  
participate in the uptake of further iron into the cell  
(Kuhn L.C. et al., in Iron Transport and Storage, CRC  
Press, Boca Raton, Ann Arbor and Boston, 1990, p. 149).

Disruption of blood circulation deprives cells of  
35   oxygen and iron and may result in cell death. Deposition  
of iron from cell death, for example in ischemic injury  
may result in the generation of highly reactive and toxic

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superoxide or hydroxyl free radicals which can result in further tissue damage. Accordingly, the abundance of iron and its availability can greatly alter survival of damaged tissues. Rapidly proliferating cells, such as malignant cells, have an increased requirement for iron and must possess efficient mechanisms to obtain iron. Limiting the ability of malignant cells to acquire iron may provide a method of killing tumor cells or of modulating their uncontrolled cell growth.

Although cellular iron uptake has been shown to be mediated mainly by the transferrin receptor (Doering, T.L. et al, J. Biol. Chem. 265:611-614, (1990), a non-transferrin-mediated pathway has been implicated for iron incorporation into cells, including leukemic cells (Basset, P. et al, Cancer Res. 46:1644-1647, 1986), HeLa cells (Sturrock, A. et al, J. Biol. Chem. 265:3139-3145, 1990), hepatocytes (Thorstensen, K., J. Biol. Chem. 263:16837-16841, 1988) and melanoma cells (Richardson, D.R. and Baker, E., Biochem. Biophys. Acta. 1053:1-12, 1990; Richardson, D.R. and Baker, E., Biochem. Biophys. Acta. 1091:294-302, 1991a and; Richardson, D.R. and Baker, E., Biochem. Biophys. Acta. 1093:20-28, 1991a).

p97, also known as melanotransferrin, a human melanoma-associated antigen, was one of the first cell surface markers associated with human skin cancer (Hellstrom, K.E. and Hellstrom, I. (1982) in Melanoma Antigens and Antibodies, Ed. Reisfield, R. and Ferrone, S., Plenum Press, New York, pp187-341). p97 is a monomeric membrane-associated protein with a molecular mass of 97,000 daltons (Brown, J.P. et al. J. Immunol. 127:539, 1981) and has been suggested as a melanoma specific marker (Estin, C.D. et al., Proc. Nat. Acad. Sci. U.S.A. 85:1052-1056, 1988). As well as being associated with the cell surface of melanomas and some other tumors and cell lines (Brown, J.P. et al., Proc. Nat. Acad. Sci. U.S.A. 78:539, 1981), p97 has also been found in certain fetal tissue (Woodbury, R.G. et al., Int. J. Cancer



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27:145, 1981) and, more recently on endothelial cells of the human liver (Sciot, R., et al., Liver 9:110, 1989).

The primary structure of p97, deduced from its mRNA sequence indicates that it belongs to a group of closely related iron binding proteins found in vertebrates (Rose, T.M. et al., Proc. Nat. Acad. Sci. U.S.A. 83:1261, 1986). This family includes serum transferrin, lactoferrin and avian egg white ovotransferrin. Human p97 and lactoferrin share 40% sequence homology ( Baker, E.N. et al., Trends Biochem. Sci. 12:350, 1987), however, in contrast to the other molecules of the transferrin family, p97 is the only one which is directly associated with the cell membrane. The deduced sequence of p97 has, in addition to a transferrin-like domain, a hydrophobic segment at its C terminal which was thought to allow the molecule to be inserted into the plasma membrane (Rose, T.M. et al., Proc. Nat. Acad. Sci. USA 77:6114, 1980).

Detergent-solubilized p97 has been reported to bind iron (Doering, T.L. et al., J.Biol. Chem. 265:611-614, 1990). However, the role of p97 in iron transport is far from clear. Iron binding to p97 at the plasma membrane has not been demonstrated and, despite numerous studies, no evidence of a role for p97 in iron mediated transport has been obtained to date. Recent studies have concluded that p97 does not play a role in iron transport (Richardson, D.R. and Baker, E. Biochem. Biophys. Acta. 1103:275-280, 1992; Richardson, D.R. and Baker, E. Biochem. Biophys. Acta. 1093:20-28, 1991 and; Richardson, D.R. and Baker, E. Biochem. Biophys. Acta. 1091:294-302, 1991). The physiological role of p97 in normal and malignant cells has not been determined.

Alzheimer's Disease has become a significant health care problem due to increases in number and longevity of the elderly. In the near future, it is predicted that a significant proportion of the elderly population may be affected. The incidence of Alzheimer's Disease increases sharply from 1% at age 65, to over 20% at age 80. After

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age 85, nearly half of the population in the United States meets the diagnostic criteria for Alzheimer's Disease (Evans et al, J.A.M.A. 262:2551-2556, 1989).

There are two alternative "criteria" which are  
5 utilized to clinically diagnose Alzheimer's Disease: the  
DSM-IIIR criteria and the NINCDS-ADRDA criteria (which is  
an acronym for National Institute of Neurological and  
Communicative Disorders and Stroke (NINCDS) and the  
Alzheimer's Disease and Related Disorders Association  
10 (ADRDA); see McKhann et al., Neurology 34:939-944, 1984).  
Briefly, the criteria for diagnosis of Alzheimer's Disease  
under DSM-IIIR include (1) dementia, (2) insidious onset  
with a generally progressive deteriorating course, and (3)  
exclusion of all other specific causes of dementia by  
15 history, physical examination, and laboratory tests.  
Within the context of the DSM-IIIR criteria, dementia is  
understood to involve "a multifaceted loss of intellectual  
abilities, such as memory, judgement, abstract thought,  
and other higher cortical functions, and changes in  
20 personality and behaviour." (DSM-IIIR, 1987).

In contrast, the NINCDS-ADRDA criteria sets forth  
three categories of Alzheimer's Disease, including  
"probable," "possible," and "definite" Alzheimer's  
Disease. Clinical diagnosis of "possible" Alzheimer's  
25 Disease may be made on the basis of a dementia syndrome,  
in the absence of other neurologic, psychiatric or  
systemic disorders sufficient to cause dementia.  
Criteria for the clinical diagnosis of "probable"  
Alzheimer's Disease include (a) dementia established by  
30 clinical examination and documented by a test such as the  
Mini-Mental test (Foldstein et al., J. Psych. Res. 12:189-  
198, 1975); (b) deficits in two or more areas of  
cognition; (c) progressive worsening of memory and other  
cognitive functions; (d) no disturbance of consciousness;  
35 (e) onset between ages 40 and 90, most often after age 65;  
and (f) absence of systemic disorders or other brain diseases  
that could account for the dementia. The criteria for

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definite diagnosis of Alzheimer's Disease include histopathologic evidence obtained from a biopsy, or after autopsy. Since confirmation of definite Alzheimer's Disease requires histological examination from a brain  
5 biopsy specimen (which is often difficult to obtain), it is rarely used for early diagnosis of Alzheimer's Disease.

Neuropathologic diagnosis of Alzheimer's Disease is typically based upon the numbers of plaques and tangles in the neurocortex (frontal, temporal, and parietal lobes),  
10 hippocampus and amygdala (Khachaturian, Arch. Neurol. 42:1097-1105; Esiri, "Anatomical Criteria for the Biopsy diagnosis of Alzheimer's Disease," Alzheimer's Disease, Current Research in Early Diagnosis, Becker and Giacobini (eds.), pp. 239-252, 1990). A diagnosis of Alzheimer's  
15 Disease based upon neuropathologic criteria alone, however, is often difficult because there are a significant number of plaques and tangles in the neurocortex, hippocampus, and amygdala of normal elderly people. In addition, the density of neocortical plaques  
20 and tangles has only a rough correlation with the degree of dementia.

Some researchers have suggested the use of quantitative electroencephalographic analysis (EEG) to diagnose Alzheimer's Disease. This method employs Fourier  
25 analysis of the beta, alpha, theta, and delta bands (Riekkinen et al., "EEG in the Diagnosis of Early Alzheimer's Disease," Alzheimer's Disease, Current Research in Early Diagnosis, Becker and Giacobini (eds.), pp. 159-167, 1990) in order to arrive at diagnosis of  
30 Alzheimer's Disease. This method, however, produces results which are difficult to interpret without control data (such as a routine EEG) from the very same patient prior to onset of Alzheimer's Disease.

Other researchers have attempted to diagnose  
35 Alzheimer's Disease by quantifying the degree of neural atrophy, since such atrophy is generally accepted as a consequence of Alzheimer's Disease. Examples of these

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methods include computed tomographic scanning (CT), and magnetic resonance imaging (MRI) (Leedom and Miller, "CT, MRI, and NMR Spectroscopy in Alzheimer's Disease," Alzheimer's Disease, Current Research in Early Diagnosis, 5 Becker and Giacobini (eds.), pp. 297-313, 1990). Although these methods show promise, they cannot yet be utilized to reliably differentiate Alzheimer's patients from normal elderly people (Bird, Prog. Neurobiol. 19:91-115, 1982; Wilson et al., Neurology 32:1054-1057, 1982; Yerby et al., 10 Neurology 35:1316-1320, 1985; Luxenberg et al., J. Neurol. Sci. 13:570-572, 1986; and Friedland et al., Ann. Int. Med. 109:298-311, 1988).

Other researchers have noticed that patients with Alzheimer's Disease often exhibit decreased cerebral blood 15 flow or metabolism in the posterior temporoparietal cerebral cortex. These researchers have therefore attempted to measure decreased blood flow or metabolism by positron emission tomography (PET) (Parks and Becker, "Positron Emission Tomography and Neuropsychological 20 Studies in Dementia," Alzheimer's Disease's, Current Research in Early Diagnosis, Becker and Giacobini (eds.), pp. 315-327, 1990), single photon emission computed tomography (SPECT) (Mena et al., "SPECT Studies in Alzheimer's Type Dementia Patients," Alzheimer's Disease, 25 Current Research in Early Diagnosis, Becker and Giacobini (eds.), pp. 339-355, 1990), and xenon inhalation methods (Jagust et al., Neurology 38:909-912; Prohovnik et al., Neurology 38:931-937; and Waldemar et al., Senile Dementias: II International Symposium, pp. 399-407, 1988). 30 These methods, however, are apparently insensitive to damage in structures such as the hippocampus and amygdala, which are believed to be the sites of damage in the earliest stages of Alzheimer's Disease's. Therefore, patients may exhibit significant memory loss, and yet 35 exhibit no abnormalities in cerebral blood flow or metabolism.

Various researchers have also attempted to

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immunologically diagnose Alzheimer's Disease (Wolozin, "Immunochemical Approaches to the Diagnosis of Alzheimer's Disease," Alzheimer's Disease, Current Research in Early Diagnosis, Becker and Giacobini (eds.), pp. 217-235, 5 1990). Wolozin and coworkers (Wolozin et al., Science 232:648-650, 1986) produced a monoclonal antibody "Alz50," that reacts with a 68-kDa protein "A68," which is expressed in the plaques and neuron tangles of patients with Alzheimer's Disease. Using the antibody Alz50 and 10 Western blot analysis, A68 was detected in the cerebral spinal fluid (CSF) of some Alzheimer's patients and not in the CSF of normal elderly patients (Wolozin and Davies, Ann. Neurol. 22:521-526, 1987). This method, however, is not presently suitable as a definitive method for 15 diagnosing Alzheimer's Disease because detectable levels of A68 could not be found in all patients with "probable" Alzheimer's Disease (as defined above).

Some researchers have attempted to identify genetic markers for Alzheimer's Disease. While genetic abnormality 20 in a few families has been traced to chromosome 21 (St. George-Hyslop et al., Science 235:885-890, 1987), such markers on chromosome 21 have not been found in other families with early and late onset of Alzheimer's Disease (Schellenberg et al., Science 241:1507-1510, 1988).

25 Others have attempted to identify neurochemical markers of Alzheimer's Disease. Neurochemical markers which have been associated with Alzheimer's Disease include reduced levels of acetylcholinesterase (Giacobini and Sugaya, "Markers of Cholinergic Dysfunction in 30 Alzheimer's Disease," Alzheimer's Disease, Current Research in Early Diagnosis, Becker and Giacobini (eds.), pp. 137-156, 1990), reduced somatostatin (Tamminga et al., Neurology 37:161-165, 1987), a negative relation between serotonin and 5-hydroxyindoleacetic acid (Volicer et al., 35 Arch Neurol. 42:127-129, 1985), greater probenecid-induced rise in homovanillic acid (Gibson et al., Arch. Neurol. 42:489-492, 1985) and reduced neuron-specific enolase

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(Cutler et al., Arch. Neurol. 43:153-154, 1986). None of these markers, however, is believed to be sensitive or specific enough to provide an early diagnosis of Alzheimer's Disease (see Elby, "Early Diagnosis of Alzheimer's Disease," Alzheimer's Disease: Current Research in Early Diagnosis, Becker and Giacobini (eds.), Taylor & Francis (pub.), N.Y., pp. 19-30, 1990).

Alzheimer's Disease has been difficult to not only diagnose, but to treat. The discovery that levels of acetylcholinesterase are markedly reduced in the cortex and hippocampus of patients with Alzheimer's Disease (Bowen et al., Brain 99:459-496, 1976) has resulted in the development of a number of pharmaceutical compounds which restore or replace cholinergic function. Examples of such compounds include tacrine (THA) (Summers et al., N. Eng. J. Med. 315:1241-1245); oral administration of choline and lecithin (Etienne et al. Neurology 31:1552-1554, 1981); huperzine A and B (Tank et al., "Studies on the Nootropic Effects of Huperzine A and B: Two Selective AChE Inhibitors," Current Research in Alzheimer's Therapy, Giacobini and Becker (eds.), pp. 289-393, 1988); galanthamine (Domino, "Galanthamine: Another Look at an Old Cholinesterase Inhibitor," Current Research in Alzheimer's Therapy, Giacobini and Becker (eds.), pp. 295-303, 1988); methanesulfonyl fluoride (Moss et al., "Methanesulfonyl Fluoride: A CNS Selective Cholinesterase Inhibitor," Current Research in Alzheimer's Therapy, Giacobini and Becker (eds.), pp. 305-314, 1988); physostigmine, an irreversible inhibitor of acetylcholinesterase (Johns et al., Banbury Report 15:435-449, 1983); and physostigmine derivatives (Brufani et al., "From Physostigmine to Physostigmine Derivatives as New Inhibitors of Cholinesterase," Current Research in Alzheimer's Therapy, Giacobini and Becker (eds.), pp. 343-352, 1988). In general, however, these compounds have met with only limited success.

Given the increasing number of individuals with

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Alzheimer's Disease, it is critical that new methods for monitoring and treating the disease be discovered. The present invention provides methods for monitoring Alzheimer's Disease, as well as methods and compositions for treating Alzheimer's Disease. These methods and compositions overcome disadvantages of prior methods and compositions, and further provide other related advantages.

#### SUMMARY OF THE INVENTION

10 The present inventors have surprisingly found that p97 is a GPI-anchored protein. The GPI-anchored protein may be reacted with an enzyme that cleaves at the GPI-anchor to provide a cleaved GPI-anchored p97 protein. The cleaved p97 can be prepared using a novel semi-continuous process. Other cleaved GPI- anchored proteins can also be prepared using the novel semi-continuous process.

The present inventors have also unexpectedly found a soluble form of p97. This soluble form is hydrophilic and is present exclusively in the aqueous phase after Triton -  
20 X-114 phase separation; it does not contain ethanolamine, and it has a slower rate of transport than GPI-anchored p97. The soluble form of p97 may be present in biological fluids such as cerebrospinal fluid (CSF), blood, or urine.

The present inventors have also shown that p97 is  
25 involved in iron transport. GPI-anchored p97 expressed on the cell surface has been shown to bind iron and bound iron is released after phospholipase treatment. p97 and transferrin were also found to be expressed in brain capillary endothelial cells in normal controls and  
30 pathological brains. Most of the p97 molecule is intracellular and its expression is coincidental with the transferrin receptor. EM also indicates that p97 crosses the blood brain barrier. p97 has also been shown to bind to a soluble form of transferrin receptor. Results of  
35 affinity chromatography experiments suggest that there is a receptor which co-recognizes p97 and the transferrin receptor.

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These findings suggest that p97 may be used to modulate iron uptake in cells. Iron uptake in cells could be modulated by varying the concentration of p97, inhibiting p97 binding to iron or to the transferrin receptor, or inhibiting binding to the receptor which co-recognizes p97 and the transferrin receptor. Accordingly, p97, and stimulants, agonists or antagonists of p97 may be useful in the treatment of conditions where there is a disturbance in iron metabolism. For example, such substances may be useful in the treatment of conditions such as haemochromatosis, neurodegenerative diseases, ischemic tissue damage, including ischemic stroke or trauma, heart disease, and tumors, in particular skin cancer.

The finding of a role for p97 in iron transport, and in particular the finding that p97 can cross the blood brain barrier, suggests that p97 can be used to transport substances such as therapeutic agents across the blood brain barrier.

The present inventors have also significantly found that reactive microglial cells associated with senile plaques in Alzheimer's Disease express p97 and transferrin receptor. Therefore, p97 and transferrin receptor can be used in the diagnosis of Alzheimer's Disease. The finding that microglial cells which deposit the amyloid protein have a high level of proteins which operate in procurement of iron also suggests methods of treatment of Alzheimer's disease based on depletion of iron from these cells using substances such as p97, transferrin, and iron chelators, for example, lactoferrin, ferritin, ovotransferrin.

Broadly stated the present invention relates to a GPI-anchored form of p97 and derivatives thereof. The invention also contemplates methods of preparing p97 and derivatives thereof.

Within one embodiment of the present invention methods are provided for preparing a cleaved form of the GPI-anchored p97, comprising incubating a cell which



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expresses p97 on its surface with an enzyme that cleaves glycosyl-phosphatidylinositol (GPI) anchors to produce the cleaved form of the GPI-anchored p97, and isolating the cleaved form. Within the context of the present invention, phospholipase cleaved p97 or cleaved p97 refers to p97 which has been cleaved from its glycosyl-phosphatidylinositol (GPI) anchor.

Preferably, a semi-continuous process for preparing cleaved GPI-anchored proteins such as cleaved GPI-anchored p97 is utilized. The semi-continuous process comprises (a) providing a cell capable of expressing a GPI-anchored protein on its surface; (b) growing the cell under conditions suitable for the expression of the GPI-anchored protein on the cell surface; (c) incubating the cell with an enzyme which is capable of cleaving the GPI anchor to form a cleaved protein; (d) recovering the cleaved protein; and (e) repeating steps (b) to (d) until a desired amount of cleaved protein is obtained. Preferably, the cell is genetically engineered to express the GPI-anchored protein.

Within another aspect of the present invention, isolated soluble p97 is provided. The soluble form of p97 is hydrophilic; present exclusively in the aqueous phase after Triton -X-114 phase separation; it does not contain ethanolamine, and it has a slower rate of transport than GPI-anchored p97. The soluble p97 can be isolated based on its hydrophilic property.

Within yet another aspect of the present invention an isolated DNA sequence is provided which encodes truncated p97. Within various embodiments of the invention, the sequence which encodes truncated p97 consists essentially of the sequence which encodes the C-terminal domain of p97, or the sequence which encodes the N-terminal domain of p97. Also provided are recombinant expression vectors for expressing such sequences, as well as the host cells which contain these expression vectors.

Within one embodiment of the invention, the p97 is

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labelled, the label being selected from the group consisting of fluorescent molecules, enzymes, luminescent molecules, radionuclides, substances having therapeutic activity, and toxins.

5       The invention also contemplates methods of modulating iron metabolism using p97. In particular, the present invention relates to a method for treating conditions involving disturbances in iron metabolism comprising administering an iron modulating amount of p97, or a  
10       stimulant, agonist or antagonist of p97. Conditions involving disturbances in iron metabolism which may be treated using the method of the invention include haemochromatosis, neurodegenerative diseases, ischemic tissue damage, including ischemic stroke or trauma, heart  
15       disease, and tumors, in particular skin cancer.

      A substance which is a stimulant, agonist or antagonist of p97 may be identified by determining the effect of the substance on the binding activity of p97 and iron, or p97 and the transferrin receptor, or the effect  
20       of the substance on the expression of p97 in cells capable of expressing p97 including cells genetically engineered to express p97 on there surface.

      The invention therefore in one aspect relates to a method of identifying stimulants, agonists or antagonists  
25       of p97 comprising reacting a substance suspected of being a stimulant, agonist or antagonist of p97 with p97 and iron under conditions such that p97 is capable of binding to the iron; measuring the amount of p97 bound to iron; and determining the effect of the substance by comparing  
30       the amount of p97 bound to iron with an amount determined for a control. The invention also relates to a method of identifying stimulants, agonists or antagonists of p97 comprising reacting a substance suspected of being a  
35       stimulant, agonist or antagonist of p97 with p97 and transferrin receptor under conditions such that p97 is capable of binding to the transferrin receptor; measuring the amount of p97 bound to transferrin receptor; and

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determining the effect of the substance by comparing the amount of p97 bound to transferrin receptor with an amount determined for a control.

The invention also relates to a method of identifying  
5 stimulants, agonists or antagonists of p97 comprising reacting a substance suspected of being a stimulant, agonist or antagonist of p97 with a cell which expresses p97, measuring the amount of p97 expressed by the cell, and determining the effect of the substance by comparing  
10 the amount of expression of p97 with an amount determined for a control.

The invention also relates to a composition for delivering an agent across the blood brain barrier comprising p97 or a substance which is capable of  
15 specifically binding to p97, in association with the agent and a pharmaceutically acceptable carrier or diluent. The p97 or substance, preferably antibody to p97 may be conjugated to the agent or a p97 fusion protein may be used in the composition. The agent may be a substance  
20 having therapeutic activity such as a growth factor or lymphokine. The invention also relates to a method of delivering an agent across the blood brain barrier comprising administering the agent in association with p97 or antibody to p97.

25 Within one aspect of the present invention, a composition for the preservation of organs intended for transplantation is provided comprising p97 or a derivative thereof in a pharmaceutically acceptable organ preservation solution. The invention also contemplates a  
30 method for preserving an organ intended for transplantation using the composition.

The present invention also provides methods for diagnosing and monitoring Alzheimer's Disease, as well as compositions and methods suitable for treating Alzheimer's  
35 Disease. Within one aspect of the present invention, methods are provided for monitoring Alzheimer's Disease, comprising detecting the presence of soluble p97 in a

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patient. Within various embodiments, the p97 may be detected in various bodily fluids, including for example, urine, blood and cerebral spinal fluid. Various methods may be utilized to detect p97, including, for example, 5 radioimmunoassays, competitive assays, and enzyme linked immunosorbant assays (ELISA) such as the sandwich assay. Within other aspects of the present invention, methods are provided for monitoring Alzheimer's Disease comprising detecting the presence of transferrin receptors, and/or 10 detecting the presence of p97, on microglial cells associated with amyloid plaques in a patient.

The invention also contemplates a bispecific antibody capable of binding to a microglial cell which expresses p97 and/or transferrin receptor and to a label preferably 15 a detectable substance, or a substance having toxic or therapeutic activity. The bispecific antibody may be prepared by forming a hybrid hybridoma from a fusion between a first cell line which produces a first monoclonal antibody which is capable of binding to a 20 microglial cell which expresses p97 and/or transferrin receptor and a second cell line which produces a second monoclonal antibody which is capable of binding to the label.

The invention further contemplates a tetrameric 25 immunological complex of a first monoclonal antibody which is capable of binding to a microglial cell which expresses p97 and/or transferrin receptor and a second monoclonal antibody which is capable of binding to a label preferably a detectable substance or a substance having toxic or 30 therapeutic activity wherein the first and second antibody are from a first animal species, conjugated to form a cyclic tetramer with two monoclonal antibodies of a second animal species directed against the Fc-fragment of the antibodies of the first animal species.

35 The tetrameric immunological complex may be formed by reacting a first monoclonal antibody which is capable of binding to a microglial cell which expresses p97 and/or

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transferrin receptor and a second monoclonal antibody which is capable of binding to a label preferably a detectable substance or a substance having toxic or therapeutic activity wherein the first and second antibody  
5 are from a first animal species, with an about equimolar amount of antibodies of a second animal species which are directed against the Fc-fragments of the antibodies of the first animal species and isolating the tetrameric complex formed.

10 The bispecific antibodies and tetrameric antibody complexes of the invention when coupled with a detectable substance may be used to identify microglial cells associated with Alzheimer's Disease.

The present invention also relates to a method of  
15 treating Alzheimer's Disease in a patient comprising depleting iron in the brain, preferably the microglial cells of the patient. In a preferred method of the invention, the treatment comprises administering p97, transferrin, transferrin receptor, or substances which are  
20 capable of reacting with p97 or transferrin receptor, preferably antibodies to p97 and transferrin or iron chelators. Exemplary iron chelators are lactoferrin, ferritin, and ovotransferrin.

Within another aspect of the present invention, a  
25 method for treating Alzheimer's Disease is provided comprising the step of administering to a patient labelled p97 or a substance which is capable of binding to p97 conjugated to a label. In one embodiment a labelled antibody to p97, or a bispecific antibody complex or  
30 tetrameric antibody complex specific for a label and p97, and which are conjugated to the label, may be administered. The label may be a toxin selected from the group consisting of ricin, abrin, diphtheria toxin, cholera toxin, gelonin, pokeweed antiviral protein, tritin,  
35 Shigella toxin, and Pseudomonas exotoxin A.

Within another aspect of the present invention a method for treating Alzheimer's Disease is also provided

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comprising the step of administering to a patient a transferrin receptor blocking agent. Examples of transferrin receptor blocking agents include a transferrin receptor blocking antibody and transferrin. An antibody  
5 to the transferrin receptor conjugated to a label as described herein or a bispecific antibody complex or a tetrameric antibody complex specific for the transferrin receptor and the label, and which is conjugated to the label, may also be used to treat Alzheimer's Disease.

10 Within another aspect of the present invention, methods are provided for treating Alzheimer's Disease comprising administering an antibody which blocks the binding of p97 to iron. Within one embodiment, the antibody is a human antibody.

15 The invention also contemplates a method of purifying microglial cells associated with Alzheimer's Disease beta amyloid plaques comprising reacting a sample suspected of containing microglial cells associated with Alzheimer's Disease beta amyloid plaques with a substance which is  
20 capable of specifically binding p97 or transferrin receptor under conditions such that the microglial cells bind to the substance; and isolating the microglial cells bound to the substance. The isolated cells may be transformed to produce a cell line. The cell line may be  
25 used to test for substances which affect the microglial cells associated with Alzheimer's Disease beta amyloid plaques. Accordingly, substances may be identified which are effective in the treatment of Alzheimer's Disease.

30 These and other aspects of the present invention will become evident upon reference to the following detailed description and attached drawings. In addition, reference is made herein to various publications, which are hereby incorporated by reference in their entirety.

#### BRIEF DESCRIPTION OF THE DRAWINGS

35 Figure 1 depicts the structure of a GPI anchor.  
Figure 2A-F depicts the nucleic acid sequence of p97.

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Figure 3 schematically depicts pWJ218.

Figure 4 schematically depicts plasmid D5-9(+).

Figure 5 is a series of graphs which show the release of p97 by PI-PLC treatment as measured by flow cytometry.

5     Figure 6 is two Western Blots which show the effect of bacterial PI-PLC on p97 expressed at the surface of SK-MEL-28 cells and a cell line transfected with the human p97 cDNA.

Figure 7 is a Western Blot which indicates the results of labelling p97 with [<sup>3</sup>H]-ethanolamine.

Figure 8 is a Western Blot which indicates the results of labelling p97 with [<sup>3</sup>H]-ethanolamine.

Figure 9 is a Western Blot which indicates the results of a phase separation of p97 and TR in Triton X-114 solution.

Figure 10 is a series of graphs which represent FACS analysis of SK-MEL-28, WTB p97aWTBc 3 and TRVb-1 cell lines stained with no primary antibody (control), L235, and OKT9.

20     Figure 11 is a series of autoradiograms which shows the effect of biosynthetic labelling on SK-MEL-28, WTB, and p97aWTBc 3 cells.

Figure 12 is an autoradiogram which shows the results of an [<sup>35</sup>S]-Methionine pulse-chase experiment.

25     Figure 13 is an autoradiogram which shows acquisition of Endo H digestion resistance during transport of p97 and TR in SK-MEL-28 cells.

Figure 14 is an autoradiogram which shows that in Triton X-114 the secreted form of p97 partitions in the aqueous phase.

Figure 15 is an autoradiogram which shows the results of biosynthetically labelling p97 with [<sup>3</sup>H]-ethanolamine followed by Triton X-114 separation.

Figure 16 is an autoradiogram which shows that membrane associated TR and p97 molecules expressed on cell surface are not released.

Figure 17 is a series of photographs of sections of

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human brain tissue showing immunohistochemical staining for p97, transferrin and transferrin receptor.

Figure 18 is electron micrographs showing sections of human brain labelled with L235 antibody.

5        Figure 19A is a photograph of a section of an Alzheimer's Disease brain, stained with anti-p97 and anti- $\beta$ , amyloid antibodies.

10       Figure 19B is a photograph of a section of an Alzheimer's Disease brain, stained with anti-p97 antibodies.

Figure 19C is a photograph of a section of negative control brain, stained with anti-p97 antibodies.

15       Figure 19D is a photograph of a section of endothelia from an Alzheimer's Disease brain, stained with anti-p97 antibodies.

Figure 19E is an enlargement of Figure 19D.

Figure 19F is a photograph of a section of a microglial cell stained with anti-p97 antibodies.

Figure 19G is an enlargement of Figure 19F.

20       Figure 19H is a photograph of a section of an Alzheimer's Disease brain, stained with anti-p97 and anti- $\beta$ -amyloid antibodies.

25       Figure 19I is a photograph of an adjacent section of the Alzheimer's Disease brain shown in Figure 19H, stained with anti-HLA-DR and anti- $\beta$ -amyloid antibodies.

Figure 19J is a photograph of an Alzheimer's Disease brain section stained with anti-p97 antibodies.

30       Figure 19L is a photograph of an Alzheimer's Disease brain section stained with anti-p97 antibodies, and no PI-PLC treatment.

Figure 19M is a photograph of an Alzheimer's Disease brain section treated with PI-PLC prior to staining with anti-p97 antibodies.

35       Figure 19N is a photograph of an Alzheimer's Disease brain section stained with anti-p97 adsorbed, anti-p97 antibodies (ie., non-reactive with p97).

Figure 20 shows two Western Blots of Alzheimer's



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Disease brain membrane and cytoplasmic samples, and SK-MEL-28, WTB and p97aWTBc3 cells stained with either L235 antibody or, no first antibody control.

Figure 21 is a series of autoradiograms showing the detection of p97 in cerebrospinal fluid of Alzheimer's Disease patients.

Figure 22 is a series of autoradiograms showing soluble and membrane bound p97 and transferrin receptor.

Figure 23 shows fluorescent labelling of CHO cells labelled with L235 and secondary fluorescinated antibody.

Figure 24 is a graph of batch growth of CHO cells showing cell concentration and glucose consumption as a function of time.

Figure 25 is a graph showing batch CHO cell protein expression as a function of time

Figure 26 is a graph showing removal of p97 from the cell surface as a function of PI-PLC concentration.

Figure 27 is a graph showing recovery of p97 expression after PI-PLC treatment.

Figure 28 is a graph showing cumulative cell specific protein release by PI-PLC as a function of harvest cycle.

Figure 29 is a graph showing cell viability, cell density, cell specific p97 harvested and, cell specific glucose uptake rate assayed at the end of each 48 hour harvest cycle.

Figure 30 is a graph showing p97 recovery in PI-PLC solution for 48 hour harvest cycle.

Figure 31 is a photograph of an SDS-polyacrylamide gel showing p97 harvested with PI-PLC and p97 released directly into the medium.

Figure 32 is a graph showing the counts per minute of [<sup>55</sup>Fe] associated with the TRVB, TRVB-1 and p97TRVB cell lines.

Figure 33 is two graphs showing the counts per minute of [<sup>55</sup>Fe] associated with the TRVB, TRVB-1 and p97TRVB cell lines before (A) and after (B) PI-PLC treatment.

Figure 34 is an autoradiogram showing the

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purification of p97 by affinity chromatography.

Figure 35 is an autoradiogram showing that p97 is resistant to Endo-H digestion.

#### DETAILED DESCRIPTION OF THE INVENTION

5       As hereinbefore mentioned the present inventors have surprisingly found that membrane-bound p-97 is a GPI-anchored protein. Accordingly, the present invention provides a GPI-anchored p-97 which is associated with the plasma membrane. The GPI-anchored p-97 is characterised  
10 in that it is sensitive to enzymes known to cleave GPI-anchors, and therefore a cleaved form of p97 can be removed from the membrane using enzymes such as bacterial PI-PLC. The present invention therefore also provides a method for isolating a phospholipase cleaved form of p-97  
15 from the cell surface by cleavage with an enzyme which is capable of cleaving a GPI-anchor, preferably PI-PLC. The presence of the GPI anchor may be shown by sensitivity to PI-PLC, insensitivity to pronase, partitioning behaviour in the detergent phase of Triton X-114 and metabolic  
20 labelling with [<sup>3</sup>H] ethanolamine.

The present inventors have also surprisingly found a soluble form of p-97. The soluble form of p-97 is present exclusively in the aqueous phase after Triton-X114 phase separation and does not contain a GPI-anchor or  
25 ethanolamine. Cell surface biotinylation of membrane bound p-97 confirmed that GPI-anchored p-97 is not shed in soluble form into the medium and that p-97 exists in two different forms, a membrane-bound form and a soluble form. The surprising discovery of a soluble form of p-97 suggests  
30 a role for soluble p-97 in binding iron in solution and then mediating its uptake via a receptor system, similar to the transferrin receptor system.

The biological activity of the membrane-bound, soluble and phospholipase cleaved forms of p97 and  
35 derivatives thereof, may be readily established by one of ordinary skill in the art by, for example, iron binding assays. For example, the biological activity of soluble

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p97 may be determined by titrating aliquots of iron (ferric nitrilotriacetate or "FeNTA") into solutions containing 1.2 mg/ml iron-free p97 in 0.025 M Tris-HCl, 0.01 M NaHCO<sub>3</sub>, 0.1 M NaCl, pH 7.8. Iron binding to soluble

5 p97 may be determined by an increase in adsorbance (measured at 420 nm). Within another embodiment, the biological activity of p97 which is anchored to a cell membrane may also be determined (see Brown et al., Nature 296:171-173, 1982). Briefly, within one embodiment

10 melanoma cells (e.g., SK-MEL-28) are washed three times with 25 ml of phosphite-buffered saline (PBS), pH 7.2, and incubated at 37° for 1 hr. with 10 µl PBS containing 2 mM NaHCO<sub>3</sub>, 1 mM sodium citrate and 10<sup>7</sup> c.p.m. of either <sup>59</sup>FeCl<sub>3</sub> or <sup>55</sup>FeCl<sub>3</sub>. Cells are washed and then lysed in 40 ml of 20

15 mM Tris-HCl buffer, pH 8.0, containing 100 mM NaCl, 1 mM EDTA and 0.5% Nonidet-P40, supplemented with 1 mM phenylmethylsulphonyl fluoride, followed by centrifugation at 300,000 g at 4°C for one hour. An anti-p97 antibody (e.g., L235 or 96.5 as described below) is added to the

20 lysate at a concentration of about 5 µg/ml, which is then passed through a 0.2 ml column of protein-A-Sepharose CL-4B at 4°C. The column is then washed, and eluted with 2 ml of 100 mM citrate buffer (pH 5), containing 0.5% Nonidet P40. Retention of <sup>59</sup>Fe or <sup>55</sup>Fe in the column

25 indicates binding of the p97 to iron.

#### PREPARATION OF P97

As noted above, within one aspect of the present invention methods are provided for preparing a cleaved form of p97 comprising the step of incubating a cell which

30 expresses p97 on its surface with an enzyme that cleaves phospholipid anchors. Briefly, prior to the present invention, it was unknown that the p97 protein is anchored to the cell surface by a glycosyl-phosphatidylinositol (GPI) anchor (see Figure 1). Various enzymes display a

35 specificity toward GPI linkages, and thus may be utilized within the context of the present invention to cleave the

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GPI anchor. Representative examples include bacterial phosphatidyl inositol-phospholipase Cs (PI-PLCs) (see Ikezawa et al., *Methods Enzymol.* 71:731-741, 1981; Taguchi et al., *Arch. Biochem. Biophys.* 186:196-201, 1978; Low, *Methods Enzymol.* 71:741-746, 1981), eukaryotic GPI-PLCs (see Ferguson et al., *J. Biol. Chem.* 260:4963-68, 1985; Bulow et al., *FEBS Lett.* 187:105-110, 1985), and eukaryotic phospholipase Ds (GPI-PLD<sub>2</sub> or "PLD") (see Malik et al., *Biochem. J.* 240:519-527, 1986) (see generally, Ferguson and Williams, "Cell-Surface Anchoring of Proteins via Glycosyl-Phosphatidylinositol Structures," *Ann. Rev. Biochem.* 57:285-320, 1988).

A particularly preferred GPI enzyme is phospholipase C (PI-PLC) which may be obtained either from bacterial sources (see Low, "Phospholipase Purification and Quantification" *The Practical Approach Series: Cumulative Methods Index*, Rickwood and Hames, eds. IRC Press, Oxford, N.Y., N.Y., 1991; Kupe et al., *Eur. J. Biochem.* 185:151-155, 1989; Volwerk et al., *J. Cell. Biochem.* 39:315-325, 1989) or from recombinant sources (Koke et al., *Protein Expression and Purification* 2:51-58, 1991; and Henner et al., *Nuc. Acids Res.* 16:10383, 1986).

p97 may be cleaved from the surface of a variety of cells including, for example, SK-MEL-28 cells (American Type Culture Collection No. HTB 72) (see also Real et al., *PNAS USA* 85:3965-3969, 1988; and Real et al., *Can. Res.* 45:44014411, 1985), as well as cells which have been infected or transfected with a vector which expresses p97 (see below). If desired, the cleaved (solubilized) p97 may then be purified utilizing techniques which are also described in more detail below, including affinity chromatography.

The soluble form of p97 may be prepared by culturing cells which contain the soluble p97 through the log phase of the cell's growth and collecting the supernatant. Preferably, the supernatant is collected prior to the time the cells reach confluency. Soluble p97 may then be

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purified as described below, in order to yield isolated soluble p97. Methods for purifying the soluble p97 can be selected based on the hydrophilic property of the soluble p97. For example, the soluble p97 may be readily obtained  
5 by Triton X-114 Phase Separation.

Within another aspect of the present invention, p97 or derivatives thereof may be recombinantly produced. Within one embodiment, DNA which codes for p97 may be obtained by polymerase chain reaction (PCR) amplification  
10 of the p97 sequence (see generally, U.S. Patent Nos. 4,683,202, 4,683,195 and 4,800,159; see also PCR Technology: Principles and Applications for DNA Amplification, Erlich (ed.), Stockton Press, 1989). Briefly, double-stranded DNA from cells which express p97  
15 (e.g., SK-MEL-28 cells) is denatured by heating in the presence of heat stable Taq polymerase, sequence specific DNA primers such as 5' GCGGACTTCCTCGG 3' (SEQUENCE ID NO: 4) and 5' TCGCGAGCTTCCT 3' (SEQUENCE ID NO: 5), ATP, CTP, GTP and TTP. Double-stranded DNA is produced when  
20 synthesis is complete. This cycle may be repeated many times, resulting in a factorial amplification of p97 DNA. The amplified p97 DNA may then be readily inserted into an expression vector as described below.

Alternatively, DNA which codes for p97 may be  
25 isolated using the cloning techniques described by Brown et al. in UK Patent Application No. GB 2188 637. Clones which contain sequences encoding p97 cDNA have been deposited with the American Type Culture Collection (ATCC) under deposit numbers CRL 8985 (PMTp97b) and CRL 9304  
30 (pSVp97a).

Within the context of the present invention, p97 and derivatives thereof may include various structural forms of the primary protein which retain biological activity. For example, a p97 protein may be in the form of acidic or  
35 basic salts, or in neutral form. In addition, individual amino acid residues may be modified by oxidation or reduction. Furthermore, various substitutions, deletions,

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or additions may be made to the amino acid or DNA nucleic acid sequences, the net effect of which is to retain biological activity of p97. Due to code degeneracy, for example, there may be considerable variation in nucleotide  
5 sequences encoding the same amino acid sequence.

Other derivatives of p97 within the scope of this invention include conjugates of p97 along with other molecules such as proteins or polypeptides. This may be accomplished, for example, by the synthesis of N-terminal  
10 or C-terminal fusion proteins to facilitate purification or identification of p97 (see U.S. Patent No. 4,851,341, see also, Hopp et al., Bio/Technology 6:1204, 1988.) Thus, fusion proteins may be prepared by fusing through recombinant techniques the N-terminal or C-terminal of p97  
15 or other portions thereof, and the sequence of a selected protein with a desired biological function. The resultant fusion proteins contain p97 or a portion thereof fused to the selected protein. Examples of proteins which may be selected to prepare fusion proteins include lymphokines  
20 such as gamma interferon, tumor necrosis factor, IL-1, IL-2, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-11, GM-CSF, CSF-1 and G-CSF. Particularly preferred molecules include nerve growth factor and the Fc portion of immunoglobulin molecules.

25 Sequences which encode the above-described molecules may generally be obtained from a variety of sources, including for example, depositories which contain plasmids encoding sequences including the American Type Culture Collection (ATCC, Rockville Maryland), and the British  
30 Biotechnology Limited (Cowley, Oxford England). Examples of such plasmids include BBG 12 (containing the GM-CSF gene coding for the mature protein of 127 amino acids), BBG 6 (which contains sequences encoding gamma interferon), ATCC No. 39656 (which contains sequences  
35 encoding TNF), ATCC No. 20663 (which contains sequences encoding alpha interferon,) ATCC Nos. 31902 and 39517 (which contains sequences encoding beta interferon), ATCC

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No. 67024 (which contains a sequence which encodes Interleukin-1 $\beta$ ), ATCC Nos. 39405, 39452, 39516, 39626 and 39673 (which contains sequences encoding Interleukin-2), ATCC Nos. 59399, 59398, and 67326 (which contain sequences  
5 encoding Interleukin-3), ATCC Nos. 57592 (which contains sequences encoding Interleukin-4). ATCC Nos. 59394 and 59395 (which contain sequences encoding Interleukin-5), and ATCC No. 67153 (which contains sequences encoding Interleukin-6).

10 Within a particularly preferred embodiment of the invention, P97 is cloned into an expression vector as a fusion gene with the constant region of human immunoglobulin  $\gamma$ 1. Briefly, the expression vectors pNUTAGH and pVL1393 are prepared for cloning by digestion  
15 with SmaI followed by dephosphorylation by calf intestinal alkaline phosphatase. The linear product is isolated after agarose gel electrophoresis. The p97 genes are then generated by polymerase chain reaction using the cloned p97 cDNA as a template. In particular, the fusion p97 is  
20 synthesized from WJ47, the 5' PCR primer encompassing coordinates 36 to 60 (coordinates based on cDNA map) and additionally containing a SnaBI restriction site. The sequence of WJ47 is 5'-GCG CTA CGT ACT CGA GGC CCC AGC CAG CCC CGA CGG CGC C-3' (Seq ID:10). The 3' primer for the  
25 fusion p97, WJ46, encompasses coordinates 2172 to 2193 and additionally contains a BclI restriction site. The sequence of WJ46 is 5'-CGC GTA CGT ATG ATC ACC CGA GCA CTG CTG AGA CGA C-3' (Seq ID:9). The resulting p97 amplified product lacks the hydrophobic domain of p97. Following  
30 amplification this product is digested with SnaBI and BclI.

The constant region of human  $\gamma$ 1 gene is then prepared from pUCB7Ig monomer. Briefly, the C $\mu$  gene is isolated by digestion with XbaI which cuts at the 3' end of the gene  
35 followed by treatment with E. coli DNA polymerase I in the presence of all four dNTPs in order to create a blunt end. The plasmid is then digested with BclI which cuts at the

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5' end of the gene. The fragment containing the heavy chain gene is isolated after electrophoresis in an agarose gel.

The fusion p97 amplified fragment is inserted into each prepared vector along with the heavy chain fragment. Orientation of the resulting plasmids is determined by PCR with one priming oligo which anneals to vector sequence and the other priming oligo which anneals to the insert sequence. Alternatively, appropriate restriction digests can be performed to verify the orientation. The sequence of the fusion p97/immunoglobulin constant region gene can be verified by DNA sequencing.

Within one embodiment of the present invention, truncated derivatives of p97 are provided. For example, site-directed mutagenesis may be performed with oligo WJ31 5'CTCAGAGGGCCGCTGCGCCC-3'(SEQ ID NO:6) in order to delete the C-terminal hydrophobic domain beyond nucleotide 2219 (see Figure 2), or with oligo WJ32 5' CCA GCG CAG CTAGCGGGGGCAG 3' (SEQ ID NO:7) in order to introduce an Nhe I site and a STOP codon in the region of nucleotides 1146-1166, and thereby also constructing a truncated form of p97 comprising only the N-terminal domain. Similarly, mutagenesis may also be performed on p97 such that only the C-terminal domain is expressed. Within one embodiment, Xho sites are inserted by mutagenesis with oligo WJ 5' ACA CCAGCGCAGCTCGAGGGGCAGCCG 3' (SEQ ID NO:8) into both the N-terminal and C-terminal domains, allowing subsequent deletion of the N-terminal domain. Various other restriction enzymes may also be utilized within the context of the present invention in order to construct deletion or truncation derivatives of p97, including for example, Eco RI.

Mutations in nucleotide sequences constructed for expression of derivatives of p97 must preserve the reading frame phase of the coding sequences. Furthermore, the mutations will preferably not create complementary regions that could hybridize to produce secondary mRNA structures,



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such as loops or hairpins, which would adversely affect translation of the receptor mRNA.

Mutations may be introduced at particular loci by synthesizing oligonucleotides containing a mutant  
5 sequence, flanked by restriction sites enabling ligation to fragments of the native sequence. Following ligation, the resulting reconstructed sequence encodes a derivative having the desired amino acid insertion, substitution, or deletion.

10 Alternatively, as noted above oligonucleotide-directed site-specific mutagenesis procedures may be employed to provide an altered gene having particular codons altered according to the substitution, deletion, or insertion required. Deletion or truncation derivatives of  
15 p97 may also be constructed by utilizing convenient restriction endonuclease sites adjacent to the desired deletion. Subsequent to restriction, overhangs may be filled in, and the DNA religated. Exemplary methods of making the alterations set forth above are disclosed by  
20 Sambrook et al. (Molecular cloning A Laboratory Manual, 2d Ed., Cold Spring Harbor Laboratory Press, 1989).

Within a particularly preferred embodiment of the invention p97 is cloned into an expression vector as a truncated gene. Briefly, the expression vectors pNUTAGH  
25 and pVL1393 are prepared for cloning by digestion with SMAI followed by dephosphorylation by calf intestinal alkaline phosphatase. The linear product of the vector is isolated after agarose gel electrophoresis. The p97 gene is then generated by polymerase chain reaction (PCR) using  
30 the cloned p97 cDNA as a template. The truncated p97 is synthesized from WJ47, the 5' PCR primer encompassing coordinates 36 to 60 (coordinates based on cDNA map) and additionally containing a SnaBI restriction site. The sequence of WJ47 is 5'-GCG CTA CGT ACT CGA GGC CCC AGC CAG  
35 CCC CGA CGG CGC C-3' (Seq ID:10). The 3' primer, WJ48, encompasses coordinates 2172 to 2193 and additionally contains both a TGA termination codon and a SnaBI

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restriction site. The DNA sequence of WJ48 is 5'-CGC GTA  
CGT ATG ATC ATC AGC CCG AGC ACT GCT GAG ACG AC-3' (Seq  
ID:11). Following amplification the truncated p97 product  
is digested with SnaBI and inserted into pNUTAGH and  
5 pVL1393 by a T4 DNA ligase reaction. Orientations of the  
resulting plasmids may be determined by PCR using one  
priming oligo which anneals to the vector sequence and the  
other priming oligo which anneals to the insert sequence.  
Alternatively, appropriate restriction digests can be  
10 performed to verify the orientation. Expression of the  
amplified sequence results in the production of a p97  
protein lacking the hydrophobic domain.

As noted above, the present invention provides  
recombinant expression vectors which include either  
15 synthetic, or cDNA-derived DNA fragments encoding p97 or  
derivatives thereof, which are operably linked to suitable  
transcriptional or translational regulatory elements.  
Suitable regulatory elements may be derived from a variety  
of sources, including bacterial, fungal, viral, mammalian,  
20 or insect genes. Selection of appropriate regulatory  
elements is dependent on the host cell chosen, and may be  
readily accomplished by one of ordinary skill in the art.  
Examples of regulatory elements include: a transcriptional  
promoter and enhancer or RNA polymerase binding sequence,  
25 a ribosomal binding sequence, including a translation  
initiation signal. Additionally, depending on the host  
cell chosen and the vector employed, other genetic  
elements, such as an origin of replication, additional DNA  
restriction sites, enhancers, sequences conferring  
30 inducibility of transcription, and selectable markers, may  
be incorporated into the expression vector.

DNA sequences encoding p97 may be expressed by a wide  
variety of prokaryotic and eukaryotic host cells,  
including bacterial, mammalian, yeast or other fungi,  
35 viral, plant, or insect cells. Methods for transforming or  
transfecting such cells to express foreign DNA are well  
known in the art (see, e.g., Itakura et al., U.S. Patent

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No. 4,704,362; Hinnen et al., PNAS USA 75:1929-1933, 1978; Murray et al., U.S. Patent No. 4,801,542; Upshall et al., U.S. Patent No. 4,935,349; Hagen et al., U.S. Patent No. 4,784,950; Axel et al., U.S. Patent No. 4,399,216; Goeddel et al., U.S. Patent No. 4,766,075; and Sambrook et al. Molecular Cloning A Laboratory Manual, 2nd edition, Cold Spring Harbor Laboratory Press, 1989, all of which are incorporated herein by reference).

Bacterial host cells suitable for carrying out the present invention include *E. coli*, *B. subtilis*, *Salmonella typhimurium*, and various species within the genus 'Pseudomonas', *Streptomyces*, and *Staphylococcus*, as well as many other bacterial species well known to one of ordinary skill in the art. Representative examples of bacterial host cells include DH5 $\alpha$  (Stratagene, LaJolla, California), JM109 ATCC No. 53323, HB101 ATCC No. 33694, and MN294.

Bacterial expression vectors preferably comprise a promoter which functions in the host cell, one or more selectable phenotypic markers, and a bacterial origin of replication. Representative promoters include the  $\beta$ -lactamase (penicillinase) and lactose promoter system (see Chang et al., Nature 275:615, 1978), the *trp* promoter (Nichols and Yanofsky, Meth in Enzymology 101:155, 1983) and the *tac* promoter (Russell et al., Gene 20: 231, 1982). Representative selectable markers include various antibiotic resistance markers such as the kanamycin or ampicillin resistance genes. Many plasmids suitable for transforming host cells are well known in the art, including among others, pBR322 (see Bolivar et al., Gene 2:9S, 1977), the pUC plasmids pUC18, pUC19, pUC118, pUC119 (see Messing, Meth in Enzymology 101:20-77, 1983 and Vieira and Messing, Gene 19:259-268, 1982), and pNH8A, pNH16a, pNH18a, and Bluescript M13 (Stratagene, La Jolla, Calif.).

Yeast and fungi host cells suitable for carrying out the present invention include, among others *Saccharomyces cerevisiae*, the genera *Pichia* or *Kluyveromyces* and various

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species of the genus *Aspergillus*. Suitable expression vectors for yeast and fungi include, among others, YC<sub>p</sub>50 (ATCC No. 37419) for yeast, and the *amdS* cloning vector pV3 (Turnbull, *Bio/Technology* 7:169, 1989). Protocols for the transformation of yeast are also well known to those of ordinary skill in the art. For example, transformation may be readily accomplished either by preparation of spheroplasts of yeast with DNA (see Hinnen et al., *PNAS USA* 75:1929, 1978) or by treatment with alkaline salts such as LiCl (see Itoh et al., *J. Bacteriology* 153:163, 1983). Transformation of fungi may also be carried out using polyethylene glycol as described by Cullen et al. (*Bio/Technology* 5:369, 1987).

Mammalian cells suitable for carrying out the present invention include, among others: COS (e.g., ATCC No. CRL 1650 or 1651), BHK (e.g., ATCC No. CRL 6281), CHO (ATCC No. CCL 61), HeLa (e.g., ATCC No. CCL 2), 293 (ATCC No. 1573) and NS-1 cells. Suitable expression vectors for directing expression in mammalian cells generally include a promoter, as well as other transcriptional and translational control sequences. Common promoters include SV40, MMTV, metallothionein-1, adenovirus Ela, CMV, immediate early, immunoglobulin heavy chain promoter and enhancer, and RSV-LTR. Protocols for the transfection of mammalian cells are well known to those of ordinary skill in the art. Representative methods include calcium phosphate mediated electroporation, retroviral, and protoplast fusion-mediated transfection (see Sambrook et al., *supra*).

Given the teachings provided herein, promoters, terminators, and methods for introducing expression vectors of an appropriate type into plant, avian, and insect cells may also be readily accomplished. For example, within one embodiment, p97 or derivatives thereof may be expressed from plant cells (see Sinkar et al., *J. Biosci (Bangalore)* 11:47-58, 1987, which reviews the use of *Agrobacterium rhizogenes* vectors; see also Zambryski et

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al., Genetic Engineering, Principles and Methods, Hollaender and Setlow (eds.), Vol. VI, pp. 253-278, Plenum Press, New York, 1984, which describes the use of expression vectors for plant cells, including, among  
5 others, pAS2022, pAS2023, and pAS2034).

Within a particularly preferred embodiment of the invention, p97 is expressed from baculoviruses, (see Example 2 below) (see also Luckow and Summers, Bio/Technology 6:47, 1988; Atkinson et al., Petic. Sci  
10 28:215-224, 1990). Use of baculoviruses such as AcMNPV is particularly preferred due to the expression of GPI-cleaved forms of p97 from the host insect cells.

p97 may be prepared by culturing the host/vector systems described above, in order to express the  
15 recombinant p97. Recombinantly produced p97 may be further purified as described in more detail below.

Alternatively, p97 may be expressed in non-human transgenic animals such as mice, rats, rabbits, sheep and pigs (see Hammer et al. (Nature 315:680-683, 1985),  
20 Palmiter et al. (Science 222:809-814, 1983), Brinster et al. (Proc Natl. Acad. Sci USA 82:44384442, 1985), Palmiter and Brinster (Cell. 41:343-345, 1985) and U.S. Patent No. 4,736,866). Briefly, an expression unit, including a DNA sequence to be expressed together with appropriately  
25 positioned expression control sequences, is introduced into pronuclei of fertilized eggs. Introduction of DNA is commonly done by microinjection. Integration of the injected DNA is detected by blot analysis of DNA from tissue samples, typically samples of tail tissue. It is  
30 preferred that the introduced DNA be incorporated into the germ line of the animal so that it is passed on to the animal's progeny. Tissue-specific expression may be achieved through the use of a tissue-specific promoter, or through the use of an inducible promoter, such as the  
35 metallothionein gene promoter (Palmiter et al., 1983, *ibid*), which allows regulated expression of the transgene. Animals which develop tissue-specific expression of p97

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(e.g., in the brain) may be utilized as disease models for Alzheimer's Disease. Alternatively, yeast artificial chromosomes (YACs) may be utilized to introduce DNA into embryo-derived stem cells by fusion with yeast spheroblasts carrying the YAC (see Capecchi, Nature 362:255-258, 1993; Jakobovits et al., Nature 362:255-258, 1993). Utilizing such methods, animals may be developed which express p97 in tissue (e.g. the brain). and which are therefore useful as a disease model for Alzheimer's Disease. Animals which do not produce p97 may be developed in order to study the function of p97.

#### CONTINUOUS PROCESS FOR PRODUCING GPI-ANCHORED PROTEINS

The present invention provides a semi-continuous process to recover heterologous proteins at increased concentrations and purities. Proteins attached to mammalian cell membranes by GPI anchors can be selectively released into the supernatant by enzymes displaying a specificity toward GPI linkages which are discussed in detail above. The present inventors have determined that this process may be repeated and used to recover increased amounts of protein. Cells may be repeatedly harvested by separating cell growth and protein expression from the enzyme treatment. The method of the invention may be carried out with a culture of cells expressing a GPI-anchored protein, preferably a cell line genetically engineered to produce the GPI anchored protein to be prepared. GPI anchored proteins which may be produced using the method of the invention include hydrolytic enzymes for example Alkaline phosphatase, 5'-Nucleotidase, Acetylcholinesterase (AChE), Trehalase, Alkaline phosphodiesterase I, gp63 proteinase, Dipeptidase, p76 proteinase, Aminopeptidase P, Lipoprotein lipase; Mammalian antigens for example, Thy-1, Thy-3, RT-6, Qa, Ly-6, MEM-43, Carcinoembryonic antigen (CEA), NCA, Blast-1, MRC OX-45, CD14, Mo3, CD48; protozoal antigens for example Ssp-4, 90 kDa glycoprotein, Variant surface

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glycoprotein (VSG) Procyclin, surface antigens, 195 kDa antigen, Transferring receptor, P30; Cell-cell interaction proteins for example, LFA-3, Heparan sulfate proteoglycan, Neural cell adhesion molecule, Contact site A, PH-20, F11; 5 and Decay accelerating factor (DAF), 130 kDa hepatoma glycoprotein, 34 kDa growth factor, scrapie prion protein, GP-2, CD16 (Fcγ receptor III), Oligodendrocyte-myelin protein, Antigen 117, 125 kDa glycoprotein C8 binding protein, Folate binding protein, Sgp-1, Sgp-2, 26 kDa 10 glycoprotein 150 kDa glycoprotein, 82 and 68 kDa proteins, surface antigens, I-Antigenic glycoprotein GP-3, preferably p97.

In a preferred embodiment of the invention CHO cells genetically engineered to express the GPI-anchored p97 15 were grown in culture. The GPI-anchored protein may be harvested by a brief incubation with an enzyme capable of cleaving the GPI anchor, such enzymes are known in the art (Ferguson, M.J., Ann. Rev. Biochem. 57:285-320, 1988) and representative examples are described above. Preferably 20 PI-PLC or GPI-PLC are used in the method of the invention. The cleaved soluble protein may be recovered from the medium and the cells returned to growth medium for further expression of the protein. Cycles of growth and harvest may be repeated until sufficient quantities of the protein 25 are obtained.

#### PURIFICATION OF P97

p97 and derivatives thereof, as well as soluble p97, may be readily purified given the teaching provided herein. Briefly, p97 may be purified either from 30 supernatants containing solubilized p97, or from cultured host/vector systems as described above. A variety of purification steps, used either alone or in combination may be utilized to purify p97. For example, supernatants obtained by solubilizing p97, or from host/vector cultures 35 as described above, may be readily concentrated using commercially available protein concentration filters, for

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example, an Amicon or Millipore Pellicon ultrafiltration unit, or by "salting out" the protein followed by dialysis. In addition to concentration, supernatants (or concentrates) may be applied to an affinity purification  
5 matrix such as an anti-p97 antibody which is bound to a suitable support. Alternatively, an anion exchange resin may be employed, for example, a matrix or substrate having pendant diethylaminoethyl (DEAE) groups. Representative matrices include acrylamide, agarose, dextran, cellulose  
10 or other types commonly employed in protein purification. Similarly, cation exchangers may be employed which utilize various insoluble matrices such as sulfopropyl or carboxymethyl groups.

Finally, one or more reversed-phase high performance  
15 liquid chromatography (RP-HPLC) steps employing hydrophobic RP-HPLC media, e.g, silica gel having pendant methyl or other aliphatic groups, can be employed to further purify a glucagon receptor composition.

Within the context of the present invention,  
20 "isolated" or "purified," as used to define the purity of p97, means that the protein is substantially free of other proteins of natural or endogenous origin, and contains less than about 1% by mass of protein contaminants due to the residual of production processes. p97 may be  
25 considered "isolated" if it is detectable as a single protein band upon SDS-PAGE, followed by staining with Coomassie Blue.

#### PREPARATION OF ANTIBODIES

Antibodies which are reactive against p97 are well  
30 known in the art. Representative examples include L235 (ATCC No. HB 8466; see Real et al., Cancer Res. 45:4401 4411, 1985), 4.1, 8.2, 96.5 and 118.1 (see Brown et al., J. Imm. 127(2):539-546, 1981; and Brown et al., PNAS USA 78(1):539-543, 1981) and 33B6E4.

35 Alternatively, p97 or derivatives thereof, soluble p97, or cells which contain p97 on their surface



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(including cells transfected with p97 DNA) may be utilized to prepare antibodies. Within the context of the present invention, antibodies are understood to include monoclonal antibodies, polyclonal antibodies, antibody fragments (e.g., Fab, and F(ab')<sub>2</sub> and recombinantly produced binding partners. Antibodies are understood to be reactive against p97 if it binds with a K<sub>d</sub> of greater than or equal to 10<sup>-7</sup> M. As will be appreciated by one of ordinary skill in the art, antibodies may be developed which not only bind to a ligand such as p97, but which also block the biological activity of the ligand (e.g, by blocking the binding of iron or transferrin receptor to p97).

Polyclonal antibodies may be readily generated by one of ordinary skill in the art from a variety of warm-blooded animals such as horses, cows, various fowl, rabbits, mice, or rats. Briefly, p97 is utilized to immunize the animal through intraperitoneal, intramuscular, intraocular, or subcutaneous injections, an adjuvant such as Freund's complete or incomplete adjuvant. Following several booster immunizations, samples of serum are collected and tested for reactivity to p97. Particularly preferred polyclonal antisera will give a signal on one of these assays that is at least three times greater than background. Once the titer of the animal has reached a plateau in terms of its reactivity to p97, larger quantities of antisera may be readily obtained either by weekly bleedings, or by exsanguinating the animal.

Monoclonal antibodies may also be readily generated using conventional techniques (see U.S. Patent Nos. RE 32,011, 4,902,614, 4,543,439, and 4,411,993 which are incorporated herein by reference; see also Monoclonal Antibodies, Hybridomas: A New Dimension in Biological Analyses, Plenum Press, Kennett, McKearn, and Bechtol (eds.), 1980, and Antibodies: A Laboratory Manual, Harlow and Lane (eds.), Cold Spring Harbor Laboratory Press, 1988, which are also incorporated herein by reference).

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Briefly, within one embodiment a subject animal such as a rat or mouse is injected with p97. The p97 may be admixed with an adjuvant such as Freund's complete or incomplete adjuvant in order to increase the resultant  
5 immune response. Between one and three weeks after the initial immunization the animal may be reimmunized with another booster immunization, and tested for reactivity to p97 using assays described above. Once the animal has plateaued in its reactivity to p97, it is sacrificed, and  
10 organs which contain large numbers of B cells such as the spleen and lymph nodes are harvested.

Cells which are obtained from the immunized animal may be immortalized by transfection with a virus such as the Epstein bar virus (EBV) (see Glasky and  
15 Reading, Hybridoma 8(4):377-389, 1989). Alternatively, within a preferred embodiment, the harvested spleen and/or lymph node cell suspensions are fused with a suitable myeloma cell in order to create a "hybridoma" which secretes monoclonal antibody. Suitable myeloma lines  
20 include, for example, NS-1 (ATCC No. TIB 18), and P3X63 - Ag 8.653 (ATCC No. CRL 1580).

Following the fusion, the cells may be placed into culture plates containing a suitable medium, such as RPMI 1640, or DMEM (Dulbecco's Modified Eagles Medium) (JRH  
25 Biosciences, Lenexa, Kansas), as well as additional ingredients, such as Fetal Bovine Serum (FBS, ie., from Hyclone, Logan, Utah, or JRH Biosciences). Additionally, the medium should contain a reagent which selectively allows for the growth of fused spleen and myeloma cells  
30 such as HAT (hypoxanthine, aminopterin, and thymidine) (Sigma Chemical Co., St. Louis, Missouri). After about seven days, the resulting fused cells or hybridomas may be screened in order to determine the presence of antibodies which are reactive against p97. A wide variety of assays  
35 may be utilized to determine the presence of antibodies which are reactive against p97, including for example Countercurrent Immuno-Electrophoresis, Radioimmunoassays,

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Radioimmunoprecipitations, Enzyme-Linked Immuno-Sorbent Assays (ELISA), Dot Blot assays, Inhibition or Competition Assays, and sandwich assays (see U.S. Patent Nos. 4,376,110 and 4,186,530; see also Antibodies: A Laboratory  
5 Manual, Harlow and Lane (eds.), Cold Spring Harbor Laboratory Press, 1988). Following several clonal dilutions and reassays, a hybridoma producing antibodies reactive against p97 may be isolated.

Other techniques may also be utilized to construct  
10 monoclonal antibodies (see William D. Huse et al., "Generation of a Large Combinational Library of the Immunoglobulin Repertoire in Phage Lambda," Science 246:1275-1281, December 1989; see also L. Sastry et al., "Cloning of the Immunological Repertoire in Escherichia  
15 coli for Generation of Monoclonal Catalytic Antibodies: Construction of a Heavy Chain Variable Region-Specific cDNA Library," Proc Natl. Acad. Sci USA 86:5728-5732, August 1989; see also Michelle Alting-Mees et al., "Monoclonal Antibody Expression Libraries: A Rapid  
20 Alternative to Hybridomas," Strategies in Molecular Biology 3:1-9, January 1990; these references describe a commercial system available from Stratacyte, La Jolla, California, which enables the production of antibodies through recombinant techniques). Briefly, mRNA is  
25 isolated from a B cell population, and utilized to create heavy and light chain immunoglobulin cDNA expression libraries in the  $\lambda$ ImmunoZap(H) and  $\lambda$ ImmunoZap(L) vectors. These vectors may be screened individually or co-expressed to form Fab fragments or antibodies (see Huse et al.  
30 supra; see also Sastry et al., supra). Positive plaques may subsequently be converted to a non-lytic plasmid which allows high level expression of monoclonal antibody fragments from E. coli.

Similarly, binding partners may also be constructed  
35 utilizing recombinant DNA techniques to incorporate the variable regions of a gene which encodes a specifically binding antibody. Within one embodiment, the genes which

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- encode the variable region from a hybridoma producing a monoclonal antibody of interest are amplified using nucleotide primers for the variable region. These primers may be synthesized by one of ordinary skill in the art, or
- 5 may be purchased from commercially available sources. Stratacyte (La Jolla, Calif) sells primers for mouse and human variable regions including, among others, primers for  $V_{H\alpha}$ ,  $V_{H\beta}$ ,  $V_{H\gamma}$ ,  $V_{H\delta}$ ,  $C_{H1}$ ,  $V_L$  and  $C_L$  regions. These primers may be utilized to amplify heavy or light chain variable
- 10 regions, which may then be inserted into vectors such as ImmunoZAP<sup>®</sup> H or ImmunoZAP<sup>®</sup> L (Stratacyte), respectively. These vectors may then be introduced into E. coli for expression. Utilizing these techniques, large amounts of a single-chain protein containing a fusion of the VH and
- 15 VL domains may be produced (See Bird et al., Science 242:423-426, 1988). In addition, such techniques may be utilized to change a "murine" antibody to a "human" antibody, without altering the binding specificity of the antibody.
- 20 Once suitable antibodies or binding partners have been obtained, they may be isolated or purified by many techniques well known to those of ordinary skill in the art (see Antibodies: A Laboratory Manual, Harlow and Lane (eds.), Cold Spring Harbor Laboratory Press, 1988).
- 25 Suitable techniques include peptide or protein affinity columns, HPLC or RP-HPLC, purification on protein A or protein G columns, or any combination of these techniques.

#### LABELLING OF P97

- p97, soluble p97, cleaved p97, and GPI-anchored p97,
- 30 and derivatives thereof, soluble p97, and antibodies which are described above may be labelled with a variety of molecules, including for example, fluorescent molecules, toxins, substances having therapeutic activity i.e. therapeutic agents, luminescent molecules, enzymes, and
- 35 radionuclides. Representative examples of fluorescent molecules include fluorescein, phycoerythrin, rhodamine,

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Texas red and luciferase. Representative examples of toxins include ricin, abrin diphtheria toxin, cholera toxin, gelonin, pokeweed antiviral protein, tritin, Shigella toxin, and Pseudomonas exotoxin A.

5 Representative examples of radionuclides include Cu-64, Ga-67, Ga-68, Zr-89, Ru-97, Tc-99m, Rh-105, Pd-109, In-111, I-123, I-125, I-131, Re-186, Re-188, Au-198, Au-199, Pb-203, At-211, Pb-212 and Bi-212. Examples of suitable enzymes include horseradish peroxidase, biotin, alkaline

10 phosphatase,  $\beta$ -galactosidase, or acetylcholinesterase; and an example of a luminescent material includes luminol. In addition, the p97 or antibodies described above may also be labelled or conjugated to one partner of a ligand binding pair. Representative examples include avidin-

15 biotin, and riboflavin-riboflavin binding protein.

Methods for conjugating or labelling the p97 or antibodies discussed above with the representative labels set forth above may be readily accomplished by one of ordinary skill in the art (see Trichothecene Antibody

20 Conjugate, U.S. Patent No. 4,744,981; Antibody Conjugate, U.S. Patent No. 5,196,951; Fluorogenic Materials and Labelling Techniques, U.S. Patent No. 4,018,884; Metal Radionuclide Labelled Proteins for Diagnosis and Therapy, U.S. Patent No. 4,897,255; and

25 Metal Radionuclide Chelating Compounds for Improved Chelation Kinetics, U.S. Patent No. 4,988,496; see also Inman, Methods In Enzymology, Vol. 34, Affinity Techniques, Enzyme Purification: Part B, Jakoby and Wichek (eds.), Academic Press, New York, p. 30, 1974; see also

30 Wilchek and Bayer, "The Avidin-Biotin Complex in Bioanalytical Applications," *Anal Biochem.* 171:1-32, 1988).

In some embodiments of the present invention, transferrin, transferrin receptor or antibodies to transferrin receptor are labelled using the techniques

35 generally known in the art and briefly mentioned above.

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A. Treatment of conditions involving disturbances of iron metabolism.

As hereinbefore mentioned, the present invention provides a method for treating conditions involving disturbances of iron metabolism by modulating p-97 mediated transport and iron uptake. p-97, agonists, antagonists and stimulants of p-97 including antibodies to p-97 and antisense to p97, may be used to modulate p97 mediated transport and iron uptake. Antibodies to p97 and their preparation have been described above. Other substances which affect p97 i.e. agonists, antagonists and stimulants of p-97 may be identified by determining the affect of the substance on the binding activity of p97 with iron or the transferrin receptor, or the affect of the substance on the expression of p97 in cells, including cells genetically engineered to express p97 such as p97aWTBc3, p97aWTBc7, and SEK-MEL-28.

The invention therefore relates to a method of identifying stimulants, agonists or antagonists of p97 comprising reacting a substance suspected of being a stimulant, agonist or antagonist of p97 with p97 and iron under conditions such that p97 is capable of binding to the iron; measuring the amount of p97 bound to iron, and determining the effect of the substance by comparing the amount of p97 bound to iron with an amount determined for a control. The method of the invention may use the iron binding assays which are described above. The p97 which may be used in the method of the invention may be the GPI-anchored p97, soluble p97, cleaved p97 or derivatives thereof, preferably recombinant p97. In the method of the invention the amount of p97 bound to iron may be determined by measuring the amount of p97 bound to iron, unbound p97 or unbound iron. p97 bound to iron may be isolated by conventional isolation techniques, for example, salting out, chromatography, electrophoresis, gel filtration, fractionation, absorption, polyacrylamide gel electrophoresis, agglutination, or combinations thereof. To facilitate the measurement of p97 bound to iron or of

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unbound p97, antibody against p97 may be utilized.

The invention also relates to a method of identifying stimulants, agonists or antagonists of p97 comprising reacting a substance suspected of being a stimulant, agonist or antagonist of p97 with p97 and transferrin receptor under conditions such that p97 is capable of binding to the transferrin receptor; measuring the amount of p97 bound to transferrin receptor; and determining the effect of the substance by comparing the amount of p97 bound to transferrin receptor with an amount determined for a control. The p97 which may be used in this method includes the GPI-anchored p97, soluble p97, or cleaved p97 or derivatives thereof, preferably recombinant p97. In the method of the invention the amount of p97 bound to transferrin receptor may be determined by measuring the amount of p97 bound to transferrin receptor, unbound p97 or unbound transferrin receptor. p97 bound to transferrin receptor may be isolated by conventional isolation techniques, for example, salting out, chromatography, electrophoresis, gel filtration, fractionation, absorption, polyacrylamide gel electrophoresis, agglutination, or combinations thereof. To facilitate the measurement of p97 bound to transferrin receptor or of unbound p97, or unbound transferrin receptor antibody against p97 or transferrin receptor which are described above may be utilized.

The invention also relates to a method of identifying stimulants, agonists or antagonists of p97 comprising reacting a substance suspected of being a stimulant, agonist or antagonist of p97 with a cell which expresses p97, measuring the amount of p97 expressed by the cell, and determining the effect of the substance by comparing the amount of p97 expression with an amount determined for a control. The p97 which may be used in this method includes the GPI-anchored p97, soluble p97, cleaved p97 or derivatives thereof, preferably recombinant p97. Cells expressing p97 which may be used in the method of the

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invention are p97aWTBc3, p97aWTBc7, and SK-MEL-28. The amount of p97 expressed on the cell may be determined by using methods known in the art, preferably labelled antibodies to p97 may be used to measure p97 expression.

5        Conditions which involve disturbances in iron metabolism which may be treated using the methods of the invention such as those involving excessive iron absorption from the diet or those requiring regular treatment by blood transfusion (e.g. dyserythropoietic  
10        anaemias, in particular thalassaemia disorders. Examples of conditions are haemochromatosis, neurodegenerative diseases (e.g. Alzheimer's Disease, Huntington's Disease and Parkinson's Disease), ischemic tissue damage, heart disease and tumors, inflammation and infections (see  
15        Pippard, J. Clinical Use of Iron Chelation, in Iron in Immunity, Cancer and Inflammation ed. M. de Sousa and J.H. Brock, 1989, John Wiley & Sons, which is incorporated herein by reference).

      Haemochromatosis is a human iron absorptive disease  
20        which involves the absorption and deposition of an excessive amount of iron which results in tissue damage (Smith, L.H., Western J. Med. 153:296-308, 1990). It is unlikely that the defect, which may be carried by as many as one in 20 individuals is a structural defect in the p97  
25        molecule because the p97 molecule is encoded on chromosome 3 in humans while the haemochromatosis gene is linked to the ferritin and HLA A genes on chromosome 6 (Zappone, E. et al., Hum. Genet. 86(6):557-61, 1991). However, the defect operates in trans and the defect may effect p97  
30        expression. The present invention provides a method for the diagnosis of haemochromatosis by assaying for increased expression of p97 by affected cells and for increased levels of soluble p97 in bodily fluids. The present invention also provides p97, or antagonists or  
35        agonists of p97 as a treatment for haemochromatosis. The efficacy of treatment may be tested in animal models for haemochromatosis, such as the hypotransferrinemic rodent



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model described in Craven, C.M. et al Proc. Nat. Acad. Sci. USA 84:3457-3461, 1987.

5 The present invention provides p-97 or antagonists or agonists of p97 as a treatment for traumatic and ischemic tissue damage, such as that resulting from heart conditions and stroke. Deposition of iron resulting from cell death may result in the generation of highly reactive and toxic superoxide or hydroxyl free radicals

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which facilitate further tissue damage. Thus the availability and abundance of iron can greatly alter the survival of damaged tissues. The efficacy of p-97 or antagonists or agonists of p97 as a treatment for  
5 traumatic and ischemic tissue damage may be tested in experiments with perfused organs, such as heart and lung, which upon transplantation suffer reperfusion injury from iron mediated generation of hydroxyl free radicals. Compounds of the invention may also be tested in animal  
10 models of heart and stroke disease, such as the Levine model (Levine, S., Amer. J. Pathol. 36:1-17, 1960) or the carbon monoxide hypoxia-oligemia model described in MacMillan V. Brain Research 151:353-368, 1978.

Rapidly proliferating malignant cells have an  
15 increased requirement for iron and must have efficient mechanisms for iron transport. An antibody-ricin conjugate prepared from a monoclonal antibody specific for transferrin receptor has been used to inhibit protein synthesis and cause cell death in a human leukemic cell  
20 line (Trowbridge, I.S. and Domingo, D.L., Cancer Surveys 1:543-556. Antibodies to transferrin receptor have also been used as pharmacological anti-tumor agents to directly block cell proliferation (Trowbridge, I.S. and Lopez, F. Proc. Nat. Acad. Sci. USA 79:1175-1179, 1982). However,  
25 anti-transferrin receptor antibodies do not significantly inhibit the growth of melanoma cells (Trowbridge, I.S. et al, Methods in Enzymol. 14:265-279.

The present invention has demonstrated that p-97, a melanoma associated antigen plays a role in the transport  
30 and cellular internalisation of iron. The present invention therefore provides a method of inhibiting protein synthesis and tumor growth and of killing tumor cells expressing p-97, such as melanoma cells, by interfering with p-97 mediated uptake of iron, for  
35 example by providing antagonists of p-97 mediated iron uptake. It is also contemplated to specifically target and kill tumor cells expressing p-97 using monoclonal

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antibodies specific to p-97, such as L235. Antibodies to p-97 may also be conjugated to a label, preferably a toxin, most preferably a cytotoxic agent. Agents cytotoxic to tumor cells which may be conjugated to antibodies are well known in the art and include conventional cytotoxic drugs such as daunomycin or adriamycin and various toxins of plant or bacterial origin such as ricin, abrin or diphtheria toxin (Trowbridge, I.S. and Domingo, D.L., Cancer Surveys 1:543-556).

10 The present invention demonstrates that the melanoma associated p-97 is involved in the transport and internalisation of iron and provides a treatment for melanoma by modulating iron transport with p-97 antagonists, antibodies directed against p97 and with  
15 other compounds effective in the removal of iron, such as iron chelators. Iron chelators are known in the art which attach ligands to iron and include lactoferrin, ferritin, porphyrin and ovotransferrin.

Within another aspect of the present invention,  
20 methods and compositions suitable for treating melanomas are provided. Briefly, as noted above, p97 was originally discovered as a cell surface marker associated with human skin cancer. Within one aspect of the present invention, a method is provided for treating skin cancer comprising  
25 administering a toxin conjugated to soluble p97. Various toxins may be conjugated to soluble p97 as described above, including, for example, bacterial exotoxins and plant toxins. Particularly preferred toxins include ricin, abrin, diphtheria toxin, cholera toxin, gelonin,  
30 pokeweed antiviral protein, tritin, Shigella toxin, and Pseudomonas exotoxin A.

Alternatively, skin cancer may also be treated or diagnosed by administering a radiolabeled soluble p97 to a patient. Briefly, the soluble p97 may be either  
35 radiolabeled directly, or conjugated to a radiolabel. Preferred radionuclides include Cu-64, Ga-67, Ga-68, Zr-89, Ru-97, Tc-99m, Rh-105, Pd-109, In-111, I-123, I-125,

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I-131, Re-186, Re-188, Au-198, Au-199, Pb-203, At-211, 20 Pb-212 and Bi-212.

#### B. Preservation of Organs

Transplantation of organs is a definitive treatment  
5 for patients with end stage liver, kidney, heart and  
pancreas disease. However, there are a number of problems  
associated with the ex vivo storage of cadaveric organs  
and thus the viability of organ transplants. One such  
10 problem is damage to the organ resulting from iron.  
Accordingly, p97 may be used in organ preservation  
solutions to control iron levels and thus improve organ  
preservation.

The present invention therefore relates to a  
composition for the preservation of an organ intended for  
15 transplantation comprising p97 or a derivative thereof in  
a pharmaceutically acceptable organ preservation solution.

The terms "preservation", or "preserving" used  
herein include but are not limited to perfusion, flushing  
and storage of an organ intended for transplantation.

20 The pharmaceutically acceptable organ preservation  
solution used in the composition of the invention may be  
any commonly used preservation solution. The ingredients  
of exemplary commonly used preservation solutions are set  
forth in U.S. Patent No. 4,920,004; Collins et al., Lancet  
25 2:1219, 1969; Sacks, S.A., Lancet 1:1024, 1973; Siegel,  
N.J. et al., Am. J. Physiol. 245:F530, 1983; Stromski,  
M.E. et al., Am. J. Physiol. 250:F834, 1986; Sumpio, B.E.  
et al., Am. J. Physiol. 247:R1047; Stromski, M.E. et al.,  
Am J. Physiol, 250:F834, 1986); Belzer et al., Transpl.  
30 Proc. 16:161, 1984; U.S. Patent No. 4,920,004; U.S. Patent  
Nos. 4,798,824 and 4,873,230; U.S. Patent No. 4,879,283;  
and U.S. Patent No. 4,879,283 (the University of Wisconsin  
solution or UW solution).

The present invention also contemplates a method  
35 for preserving an organ intended for transplantation using  
the composition described above. Generally, an organ may  
be flushed during harvesting and after its removal from

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the donor with a composition of the invention. The organ is then stored in a composition of the invention under hypothermic conditions. In the alternative, after initial flushing, the organ may be connected to a pump wherein a  
5 cold perfusate of the composition of the invention is continuously circulated through the organ. Prior to transplantation the organ may be flushed again with the composition.

The preservation method and composition of the  
10 invention may be used to preserve any organ intended for transplantation, preferably an intraabdominal organ such as the liver, pancreas and kidney.

#### C. Drug Delivery Compositions and Methods

A major obstacle to testing drugs for use in the  
15 treatment of Alzheimer's disease and other neurological conditions is the lack of an efficient non-invasive means to deliver drugs or chemotherapeutic agents across the blood brain barrier. Drug and solute transport into the brain from blood is restricted by the limited permeability  
20 of the brain capillary endothelial wall due to the endothelial tight junctions and the lack of aqueous pores in the endothelial cells (Pardridge, W.M. et al., J. Pharmacol. & Expt. Therapeut. 253:884-891, 1990). The present invention provides a mechanism for delivering  
25 blood-borne agents into the brain across the blood brain barrier. The inventors have demonstrated that p-97 is expressed on the surface of the brain capillary endothelial cells in a pattern similar to that of transferrin receptor. p-97 on the endothelial cells  
30 appears to be involved in the transport of iron across the blood brain barrier, possibly via an interaction with the transferrin receptor.

The invention contemplates a composition for delivering agents into the brain from the blood via a p-97  
35 mediated uptake mechanism. The delivery composition may contain p97 conjugated to the agent; a p97 fusion protein comprising p97 or a portion thereof fused to the agent; or

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a substance capable of binding to p97, e.g. anti-p-97 antibody, conjugated to the agent, and a pharmaceutically acceptable carrier or diluent.

p97 which may be used in the delivery compositions of  
5 the invention include soluble p97, cleaved p97, and derivatives and portions thereof. Antibodies to p97 which may be used in the delivery composition have been described above. Representative examples of p97 fusion  
10 proteins include a p97-nerve growth factor fusion protein, a p97-Ig fusion protein, or an anti-p97 antibody-nerve growth factor or Ig fusion protein.

Agents which may be used in the delivery composition of the invention are those known for the treatment of neurological conditions or suspected of having activity  
15 against neurological conditions. Accordingly, neurological conditions which may be treated using the delivery compositions of the invention include those conditions susceptible to therapeutics delivered into the brain and include, for example tumors of the brain,  
20 neurodegenerative diseases (Alzheimer's disease, Parkinson's disease, Huntington's disease), demyelinating diseases (e.g. multiple sclerosis), amyotrophic lateral sclerosis, bacterial and viral infections, and deficiency diseases (e.g. Wernicke's Disease and nutritional  
25 polyneuropathy).

Suitable cytotoxic therapeutic agents for the treatment of tumors are discussed elsewhere in the application.

Possible therapeutic agents which can be used in the  
30 compositions of the invention for the treatment of Alzheimer's disease include iron sequestering compounds, such as iron chelators, and anti-inflammatory drugs. Proteins such as growth factors, including nerve growth factor, brain-derived neurotrophic factor, and lymphokines  
35 including gamma interferon, tumor necrosis factor, the interleukins, GM-CSF, CSF-1, and G-CSF are also contemplated as therapeutic agents for use in the delivery

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compositions of the invention. Cholinergic neurons of the basal forebrain, which degenerate in Alzheimer's disease, are known to depend on nerve growth factor for their survival. Nerve growth factor has also been shown to  
5 rescue degenerating cholinergic neurons in the forebrain (Hefti, F. J. Neurosci 6:2155, 1986).

The delivery compositions may be prepared using techniques known in the art. For example, antibodies and therapeutic agents which are proteins may be conjugated by  
10 methods known in the art, such as the introduction of a sulfhydryl group on the antibody and the introduction of a cross-linker containing a reactive thiol group on to the protein agent through carboxyl groups (Wawizynczak, E.J. and Thorpe, P.E. in Immunoconjugates: Antibody Conjugates  
15 in Radioimaging and Therapy of Cancer, C.W. Vogel (Ed.) Oxford University Press, 1987, pp. 28-55.; and Blair, A.H. and T.I. Ghose, J. Immunol. Methods 59:129, 1983). A p97 fusion protein comprising p97 or a portion thereof fused to the agent may be prepared using the methods described  
20 above.

The delivery compositions of the invention may be tested for their ability to cross the blood brain barrier and provide the desired pharmacological effect using in vitro and in vivo models of the blood brain barrier.  
25 Examples of in vitro models include bovine capillary endothelial cell lines, which in culture form an endothelial monolayer with high resistance to drug and solute transport (Pardridge, W.M. et al., J. Pharmacol. & Expt. Therapeut. 253:884-891, 1990). Examples of in vivo  
30 models of the blood brain barrier include intraocular transplants of septal tissue in rats. The grafted tissue develops the endothelial and astrocytic mechanism characteristic of the blood brain barrier.

The invention also contemplates a method for  
35 delivering a selected agent across the blood brain barrier comprising administering a delivery composition of the invention containing the agent. Any route of

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administration which dilutes the composition into the blood stream could be used. Preferably, the composition is administered peripherally, most preferably intravenously or by cardiac catheter. Dosages to be  
5 administered will depend on individual needs, on the desired effect and on the chosen route of administration.

#### MONITORING ALZHEIMER'S DISEASE

The present invention provides methods for monitoring and diagnosing Alzheimer's Disease in a patient, as well  
10 as compositions and methods suitable for treating Alzheimer's Disease. These compositions and methods are based on the finding by the present inventors that p97 and transferrin receptor can be found on microglial cells associated with amyloid plaques in an Alzheimer's Disease  
15 patient and on the discovery that a soluble form of p97 may be detected in the cerebrospinal fluid of an Alzheimer's disease patient.

For the purpose of monitoring or diagnosing Alzheimer's Disease, the presence of p97 may be detected  
20 from a variety of sources in the body, including both tissues and fluids.

Within one embodiment of the invention, methods are provided for monitoring Alzheimer's Disease comprising the step of detecting the presence of either p97 or  
25 transferrin receptors on microglial cells associated with amyloid plaques in a patient. Briefly, samples may be obtained from a patient either by biopsy (e.g, computed tomographic (CT)-guided stereotactic biopsy, see Alesch et al., Acta Neurochir. (Wien) Suppl. 53: 33-36, 1991;  
30 Lazareff Acta Neurochir. (Wien) 113(1-2):82-83, 1992; Marks et al., N.Z. Med. J. 105(929):85-86, 1992; Yeo et al., Singapore Med. J. 32(5):307311, 1992), or upon autopsy, and prepared for staining according to standard histopathological procedures (see, for example, Example 8  
35 below).

Microglial cells which are associated with amyloid



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plaques may be readily identified given the disclosure provided herein (see also, Basic Histopathology, Wheator ed. Churchill Livingstone, New York; Color Atlas of Histology, Gartner ed., Williams and Wilkins, Baltimore, MD.; Histology, Ross ed., Harper and Row, San Francisco, CA.; Elbe, "Early Diagnosis of Alzheimer's Disease," Alzheimer's Disease: Current Research in Early Diagnosis, supra; Beyruther et al., "Mechanisms of amyloid deposition in Alzheimer's disease," Ann. N.Y. Acad Sci 640:129-139, 1991; Kawai et al., "Subcellular localization of amyloid precursor protein in senile plaques of Alzheimer's disease," Am. J. Pathol. 140(4):947-958, 1992).

Particularly preferred methods for identifying microglial cells which are associated with amyloid plaques are described in more detail below in Example 8. Briefly, as shown in Figure 19A, microglial cells (MC) which are stained with an anti-p97 antibody are directly associated with amyloid plaques (P). Blood vessels are identified as "BV". Staining of microglial cells with antitransferrin receptor antibodies in place of anti-p97 antibodies produces results similar to that seen in Figure 19A. Although normal microglial cells may have, for example, as many as 300-400 transferrin receptors on the cell surface, microglial cells from an Alzheimer's Disease patient usually have 5,000 or greater transferrin receptors on the cell surface. The increased numbers of transferrin receptors or p97 on microglial cells of an Alzheimer's Disease patient thus allows visualization of the microglial cell upon staining, whereas, microglial cells from a normal patient will not be stained (see Figure 19C). Therefore, it should be understood within the context of the present invention that the presence of p97 or transferrin receptors is detected, if the microglial cells may be visualized by staining with anti-p97 or anti-transferrin receptor antibodies.

Samples which have been obtained as described above may be readily stained with either anti-p97 antibodies, or

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anti-transferrin receptor antibodies. Anti-p97 antibodies such as L235 are described in more detail above. Anti-transferrin receptor antibodies may similarly either be prepared utilizing techniques similar to those described  
5 above, or obtained from commercial sources. Representative anti-transferrin receptor antibodies include OKT 9 (ATCC No. CRL 8021), SE9C11 (ATCC No. HB 21), L5.1 (ATCC No. HB 84), R17 217.1.3 (ATCC No. TIB 219), and R17 208.2 (ATCC No. TIB 220) (Cell Immunol.  
10 83:14-25, 1984; J. Cell. Physiol. 112:403-410, 1982; and Blood 59:671-678, 1982). Finally, anti- $\beta$  amyloid plaque antibodies may also be readily obtained utilizing techniques similar to those described above (see also, Allsop et al., Proc Natl. Acad. Sci USA 85:2790-2794,  
15 1988; Arai et al., Proc Natl. Acad. Sci USA 87:2249-2253, 1990; Benowitz et al., Exp. Neurol. 106:237-250, 1989; Cole et al., Neurobiol. Aging 12:85-91, 1991; Cras et al., Am. J. Patol. 137:241-246, 1990; Currie et al., Neuropathol. Exp. Neurol. 48:328, 1989; Ghiso et al,  
20 Biochem. Biophys. Res. Commun. 163:430-437, 1989; Ishii et al., Neuropathol. Appl. Neurobiol. 15:135-147, 1989; Joachim et al., Am. J. Pathol. 138:373-384, 1991; Kametani et al., Biomed. Res. 10:179-183, 1989; and Palmert, Biochem. Biophys. Res. Commun. 156:432-437, 1988).

25 Within another aspect of the present invention, methods are provided for monitoring Alzheimer's Disease, comprising the step of detecting the presence of soluble p97 in a patient. The presence of p97 may be determined from a variety of bodily fluids, including for example,  
30 urine, cerebral spinal fluid (CSF) and blood. Briefly, within one embodiment, a sample of fluid is removed from a patient and assayed for the presence of soluble p97. A variety of assays may be utilized, including for example Countercurrent Immuno-Electrophoresis (CIEP),  
35 Radioimmunoassays, Radioimmunoprecipitations, and Enzyme-Linked Immuno-Sorbent Assays (ELISA), Dot Blot assays, Inhibition or Competition assays and sandwich assays (see

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U.S. Patent Nos. 4,376,110 and 4,486,530; see also Antibodies: A Laboratory Manual, supra).

Within one embodiment, 100  $\mu$ l of an anti-p97 antibody such as L235 is incubated in a 96 well plate overnight at 37°C. The next day the plate is rinsed and then incubated with 200  $\mu$ l of 5% PBS/BSA for 30 minutes at 37°C. The plate is then washed, and 100  $\mu$ l of patient fluid serially diluted in 1% PBS/BSA (along with appropriate positive and negative controls) is placed in the wells of the plate. The plate is incubated for 1 hour at 37°C, and then washed three times with 1% PBS/BSA. One hundred microliters of another anti-p97 antibody such as 96.5 diluted in 1% PBS/BSA is then incubated at 37°C in the wells for 30 minutes, followed by three washes with 1% PBS/BSA. One hundred microliters of horse radish peroxidase goat anti-mouse IgG diluted in 1% PBS/BSA is then incubated in the well for 30 minutes, followed by three washes with 1% PBS/BSA. One hundred microliters per well of O-Phenylenediamine (OPD) substrate solution (1 mg/ml OPD (00-2003, Zymed), 0.001%  $H_2O_2$ , in 0.1 M citrate buffer pH 4.5) is added to each well. Plates may be read on a Titertek Multiscan Plate reader (Flow Laboratories) at 450 nm after 15 minutes. Presence of soluble p97 in the bodily fluid is indicated by the presence and degree of color, as compared to negative controls.

The invention also contemplates a bispecific antibody capable of binding to a microglial cell which deposits the amyloid protein and which expresses p97 and/or transferrin receptor, and to a label preferably a detectable substance such as a fluorescent molecule, luminescent molecule, enzyme, and radionuclide, representative examples of which are set out herein.

Bispecific antibodies may be prepared by forming hybrid hybridomas. The hybrid hybridomas may be prepared using the procedures known in the art such as those disclosed in Staerz & Bevan, (1986, PNAS (USA) 83: 1453) and Staerz & Bevan, (1986, Immunology Today, 7:241). In

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general, a hybrid hybridoma is formed by fusing a first cell line which produces a first monoclonal antibody which is capable of binding to a microglial cell expressing p97 and/or transferrin receptor and a second cell line which  
5 produces a second monoclonal antibody which is capable of binding to a label preferably a detectable substance. The first monoclonal antibody may be specific for p97 or transferrin receptor. The bispecific antibodies may also be constructed by chemical means using procedures such as  
10 those described by Staerz et al., (1985, Nature, 314:628) and Perez et al., (1985 Nature 316:354).

Bispecific chimeric monoclonal antibodies containing a variable region of an antibody for example, murine antibody, specific for p97 and/or transferrin receptor, a  
15 variable region of an antibody which is capable of binding to a label preferably a detectable substance and the constant regions of human immunoglobulin such as human IgG1, IgG2, IgG3 and IgG4 antibody may also be constructed as described above.

20 The invention further contemplates a tetrameric immunological complex of a first monoclonal antibody which is capable of binding to a microglial cell expressing p97 and/or transferrin receptor and a second monoclonal antibody which is capable of binding to a label preferably  
25 a detectable substance wherein the first and second antibody are from a first animal species, conjugated to form a cyclic tetramer with two monoclonal antibodies of a second animal species directed against the Fc-fragment of the antibodies of the first animal species.

30 A tetrameric immunological complex may be prepared by preparing a first monoclonal antibody which is capable of binding to a microglial cell expressing p97 and/or transferrin receptor and a second monoclonal antibody which is capable of binding to a label preferably a  
35 detectable substance. The first and second antibody are from a first animal species. The first and second antibody are reacted with an about equimolar amount of antibodies

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of a second animal species which are directed against the Fc-fragments of the antibodies of the first animal species or the Fab fragments of such antibodies. The tetrameric complex formed is then isolated. (See U.S. Patent No. 5 4,868,109 to Lansdrop for a description of methods for preparing tetrameric antibody complexes). The first monoclonal antibody may be specific for p97 or transferrin receptor.

The label should be capable of provoking the 10 production of antibodies in order to prepare the bispecific antibody and tetrameric antibody complexes of the invention. Examples of detectable substances which are capable of provoking production of antibodies are enzymes, such as horseradish peroxidase, alkaline 15 phosphatase, glucose oxidase and galactosidase. Examples of toxins which are capable of provoking the products of antibodies are radionucleotides, diphtheria toxin and ricin or attenuated derivatives thereof as described. It is also contemplated that cytotoxic cells such as macrophages, 20 neutrophils, eosinophils, NK cells, LAK cells, and large granular lymphocytes may be used as a label. It will be appreciated that the antibody may be directed against the Fc receptor on cytotoxic cells.

Bispecific antibodies and tetrameric antibody 25 complexes of the invention coupled to the label preferably a detectable substance, may be used to identify microglial cells associated with Alzheimer's Disease.

The present invention also contemplates that the above-noted methods for diagnosing and monitoring 30 Alzheimer's Disease can be used in combination with other diagnostic methods. Beta amyloid protein is internalised into cells as a conjugate with elastase. More particularly beta amyloid binding elastase may be used in combination with the methods of the present invention to target 35 diseased microglial cells.

The invention further provides a method for purifying microglial cells associated with Alzheimer's Disease beta

amyloid plaques to provide a purified population of diseased cells which may be used to test for substances which may be effective in the treatment of Alzheimer's Disease. The cell population may be purified using techniques known in the art. Preferably, the cell population is purified using a substance which is capable of specifically binding p97 or transferrin receptor. In one embodiment, the cell population is purified by affinity chromatography employing immobilised anti-p97 antibodies to selectively bind microglial cells, which have been demonstrated to express high levels of surface associated p97. The purified cell population may be transformed, to produce a cell line of Alzheimer's disease microglial cells. Macrophages may be successfully immortalised using methods known in the art, for example using SV-40 virus (Kreuzburg-Duffy, U. and MacDonald, C., Immunol. 72:368-372, 1991). Accordingly, the invention contemplates the preparation of macrophage cell lines exhibiting the elevated levels of p-97, characteristic of the diseased brain in Alzheimer's disease. This cell line will be particularly useful for further characterisation of the disease state and to provide an in vitro system for testing for substances which may have therapeutic utility in the treatment of the disease. The method may also be used to purge bone marrow cells of microglial cells associated with Alzheimer's Disease beta amyloid plaques.

It will be appreciated that the presence of p97 on the microglial cells associated with Alzheimer's Disease indicates that p97 may also be a useful marker for activated macrophages or monocytes. Accordingly, p97 may be a general indicator of disease and in particular inflammation. Thus, the above described methods and compositions for monitoring and diagnosing Alzheimer's disease may be applied to the monitoring and diagnosis of disease states and in particular inflammatory conditions such as rheumatoid arthritis, pulmonary vasculitis, allergic encephalomyelitis, allograft rejection, chemical tissue injury. (See Pippard M.J. supra).

It will also be appreciated that p97 may also be

useful in purging bone marrow of p97 positive bone marrow cells i.e. diseased cells. Thus, the methods described above for microglial cells associated with Alzheimer's Disease may be used to purge bone marrow cells.

#### TREATMENT OF ALZHEIMER'S DISEASE

As noted above, the present invention provides methods and compositions suitable for treating Alzheimer's Disease. Microglial cells have been implicated as a causative agent of Alzheimer's Disease (Schnabel, J., Science 260:1719-1720, 1993). The finding by the present inventors that microglial cells which deposit the amyloid protein have a high level of proteins i.e. p97 and transferrin receptor, which operate in procurement of iron suggests that Alzheimer's Disease may be treated by depleting iron from the microglial cells. Iron may be depleted from the microglial cells using p97, transferrin, transferrin receptor, antibodies to p97 or transferrin receptor and iron chelators such as alctoferrin, ferritin, desferrithiocin, and ovotransferrin. (See Pippard, M.J., supra).

Accordingly, within another embodiment of the present invention, a method is provided for treating Alzheimer's Disease comprising administering to a patient a transferrin receptor blocking agent. Transferrin receptor blocking agents may be readily identified by one of ordinary skill in the art given the disclosure provided herein, and including, for example, transferrin and transferrin receptor blocking antibodies. Transferrin receptor blocking antibodies may be readily prepared utilizing methods described above for making antibodies (e.g., by immunizing mice with the transferrin receptor or transferrin receptor bearing cells), and by assaying for the blocking of transferrin-transferrin receptor binding (e.g., for example, by competition assays).

Within another embodiment of the present invention, a method is provided for treating Alzheimer's Disease comprising administering to a patient an antibody which blocks the binding of p97 to iron. Antibodies which block

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the binding of p97 to iron may be readily prepared as described above (e.g., by immunizing mice with p97), and by assaying for antibodies which competitively inhibit the binding of p97 to iron.

5        Within one embodiment of the present invention, a method is provided for treating Alzheimer's Disease, comprising the step of administering to a patient labelled p97 or transferrin receptor. The transferrin receptor or p97 is preferably labelled with a toxin as described  
10 above, in order to destroy microglial cells which are associated with amyloid plaques in a patient. Representative examples of suitable toxins include ricin, abrin, diphtheria toxin, cholera toxin, gelonin, pokeweed antiviral protein, tritin, Shigella toxin, and Pseudomonas  
15 exotoxin A.

As discussed above, the present inventors have identified p97 and transferrin receptor as specific markers for microglial cells associated with beta-amyloid damaged neurons in the brain of Alzheimer's disease  
20 patients. Accordingly, the microglial cells which are associated with amyloid plaques may be targeted using substances which are capable of binding to p97 or transferrin receptor. Therefore the invention provides a method for treating Alzheimer's Disease comprising  
25 administering a substance which is capable of binding to p97 or transferrin receptor conjugated to a label, preferably a substance have therapeutic activity or a toxin as described above. The substance may be anti-p97 antibody or anti-transferrin receptor antibody,  
30 representative examples of which are described above.

The invention also contemplates a bispecific antibody capable of binding to a microglial cell which deposits the amyloid protein which expresses p97 and/or transferrin receptor, and to a label preferably, a substance having  
35 toxic or therapeutic activity. Examples of toxic substances and substances having therapeutic activity in Alzheimer's Disease are set out herein. It should be noted



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that the toxic substance may also be a cytotoxic cell as described above. The bispecific antibody should be capable of crosslinking the microglial cell and toxic substance. Where the label is a cytotoxic cell, the crosslinking of  
5 the microglial cell and the cytotoxic cell will facilitate lysis of the microglial cell.

The bispecific antibody may be prepared as described in detail above. Generally, a hybrid hybridoma is formed from a fusion between a first cell line which produces a  
10 first monoclonal antibody which is capable of binding to a microglial cell which expresses p97 and/or transferrin receptor and a second cell line which produces a second monoclonal antibody which is capable of binding to a label preferably a substance having toxic or therapeutic  
15 activity.

The invention further contemplates a tetrameric immunological complex of a first monoclonal antibody which is capable of binding to a microglial cell expressing p97 and/or transferrin receptor and a second monoclonal  
20 antibody which is capable of binding to a label preferably, a substance having toxic or therapeutic activity wherein the first and second antibody are from a first animal species, conjugated to form a cyclic tetramer with two monoclonal antibodies of a second animal species  
25 directed against the Fc-fragment of the antibodies of the first animal species.

The tetrameric immunological complex may be formed as described above. Generally, a first monoclonal antibody which is capable of binding to a microglial cell  
30 expressing p97 and/or transferrin receptor is reacted with and a second monoclonal antibody which is capable of binding to a label preferably a substance having toxic or therapeutic activity wherein the first and second antibody are from a first animal species, with an about equimolar  
35 amount of antibodies of a second animal species which are directed against the Fc-fragments of the antibodies of the first animal species and isolating the tetrameric complex

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formed.

The bispecific antibodies and tetrameric immunological complexes of the invention directed against a substance having toxic or therapeutic activity coupled  
5 with the substance having toxic or therapeutic activity may be used to treat Alzheimer's Disease. Accordingly the invention provides a composition comprising bispecific antibodies or tetrameric immunological complexes in a pharmaceutically acceptable carrier wherein the bispecific  
10 antibodies or tetrameric immunological complexes are capable of binding to a substance having toxic or therapeutic activity and to a micorglial cell expressing p97 and/or transferrin receptor.

The invention also provides a method for treating  
15 Alzheimer's Disease comprising administering to a patient in need of such treatment a therapeutically effective amount of bispecific antibodies or tetrameric immunological complexes which are specific to a substance having toxic or therapeutic activity and to microglial  
20 cells expressing p97 and/or transferrin receptor, and which are coupled to the substance and, monitoring the progress of the disease state, and, if desired, repeating the administration.

Within yet another aspect of the present invention,  
25 viral vectors may be utilized to treat Alzheimer's Disease. Briefly, within one embodiment of the invention, viral vectors may be utilized to direct the expression of antisense p97 RNA in order to prohibit expression of p97. Viral vectors suitable for use in the present invention  
30 are well known in the art including recombinant vaccinia viral vectors (U.S. Patent Nos. 4,603,112 and 4,769,330), recombinant pox virus vectors (PCT Publication No. WO 89/01973), and preferably, retroviral vectors ("Recombinant Retroviruses with Arnphtropic and Ecotropic  
35 Host Ranges," PCT Publication No. WO 90/02806; "Retroviral Packaging Cell Lines and Processes of Using Same," PCT Publication No. WO 89/07150; and "Antisense RNA for

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Treatment of Retroviral Disease States," PCT Publication No. WO 87/03451).

Therapeutic compositions of the present invention (including for example, labelled p97, labelled anti-p97  
5 antibody, p97 fusion proteins, p97 conjugated to an agent, bispecific antibodies, tetrameric antibody complexes, transferrin receptor blocking agents, and antibodies which block the binding of p97 to iron) may be administered to a patient for treatment in a manner appropriate to the  
10 indication. Typically, therapeutic compositions described above will be administered in the form of a pharmaceutical composition comprising purified protein in conjunction with physiologically acceptable carriers, excipients or diluents. Such carriers will be nontoxic to recipients at  
15 the dosages and concentrations employed. Ordinarily, the preparation of such compositions entails combining the therapeutic agent with buffers, antioxidants such as ascorbic acid, low molecular weight (less than about 10 residues) polypeptides, proteins, amino acids,  
20 carbohydrates including glucose, sucrose or dextrans, chelating agents such as EDTA, glutathione and other stabilizers and excipients. Neutral buffered saline or saline mixed with nonspecific serum albumin are exemplary appropriate diluents. The amount and frequency of  
25 administration will depend, of course, on such factors as the nature and severity of the indication being treated, the desired response, the condition of the patient, and so forth. Typically, the compositions may be administered by bolus injection, continuous infusion, sustained release  
30 from implants, or other suitable technique. Preferably, however, the pharmaceutical compositions are delivered directly into the cerebrospinal fluid.

The present invention also relates to a method of treating Alzheimer's disease by bone marrow transplant.  
35 Bone marrow transplants are performed in patients whose immune and blood forming-systems have been devastated by leukemia, cancer, chemotherapy, radiation therapy and the

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like. Stem cell transplants can also treat metabolic disorders of macrophages, such as osteopetrosis and severe Gaucher's disease (Goldie, D.W. Scientific American, December 1991, p. 86-93. Based on the present finding  
5 that microglial cells associated with beta amyloid plaques in Alzheimer's Disease brain have very high levels of expression of p97, the present invention provides a method of treating Alzheimer's Disease by bone marrow transplant to repopulate the patient with genetically altered  
10 macrophages. Microglial cells are macrophages which have populated the brain. For example, it is contemplated that a patient's own myeloid stem cells may be genetically altered to produce macrophages expressing chemotherapeutic agents in the brain after autologous transplant. Stem  
15 cells may be transformed prior to transplant to express a chemotherapeutic agent under the control of a macrophage specific promoter. Antagonists of p97 and other compounds which would deprive the cells of iron are examples of suitable chemotherapeutic agents. Suitable  
20 chemotherapeutic agents may also be selected from cytotoxic anti-tumor drugs, discussed above, and drugs which inhibit inflammation and growth. Anti-inflammatory drugs are known in the art and have been implicated in the treatment of Alzheimer's disease (Schnabel, J. Science  
25 260:1719-1720, 1993). Examples of anti-inflammatory drugs include non-steroidal anti-inflammatory compounds such as indomethacin and aspirin-like compounds.

#### MONITORING AND TREATMENT OF CONDITIONS INVOLVING ACTIVATED PERICYTES

30 An examination of the photographs of the sections of Alzheimer's disease brains stained with anti-p97 antibody appeared to show the presence of darkly stained pericytes associated with the capillary endothelial cells, suggesting that these pericytes are positive for p-97.  
35 Thus p-97 may be a marker for pericytes associated with the brain capillary endothelial cells and may also be a

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specific marker for activated pericytes and for pericytes in Alzheimer's disease brains. Interestingly, swelling of pericytes connected to brain endothelial cells has previously been associated with Alzheimer's disease.

5 Pericytes are multipotent cells closely associated with microvessel endothelial cells and are considered to be phagocytic in the central nervous system. Pericytes form close connections with endothelial cells and are thought to play a role in the regulation of epithelial  
10 cells and in capillary growth, for example in wound healing and in the vascularization of tumors. In the brain, pericytes are particularly associated with the blood brain barrier and may form a secondary line of defence by phagocytising materials which cross the blood  
15 brain barrier (Sims, D.E., Can. J. Cardiol. 7:431-443, 1991). Pericytes concentrate round endothelial cell junctions and exhibit a contractile response to inflammation. Pericytes are more numerous on brain capillary endothelial cells in Alzheimer's patients,  
20 resulting in a drastic alteration in the morphology of cerebral microvessels (Sims, D.E., Can. J. Cardiol. 7:431-443, 1991).

The present invention indicates that p97 may be a marker for pericytes, activated pericytes, tumor  
25 vascularization and Alzheimer's diseased brain and can therefore be used to monitor and diagnose conditions involving activated pericytes as described herein. It is also contemplated that the compositions of the present invention utilising p-97 described herein, preferably  
30 compositions comprising substances capable of binding to p97 conjugated to a toxin or a substance having therapeutic activity, will be useful in the treatment of conditions involving activated pericytes, such as Alzheimer's disease, diabetes, tumors with active  
35 vascularisation, inflammatory conditions and neurological disorders.

The following examples are offered by way of

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illustration, and not by way of limitation.

### EXAMPLES

#### EXAMPLE 1

##### **TRANSFECTION OF CELLS WITH P97 cDNA**

5       The Chinese Hamster Ovary ("CHO") cell lines WTB (Wild-Type) and TRVB (Transferrin Receptor Minus) (see McGraw et al., J. Cell. Biol. 105(1):207-214, 1987) were plated on 60 mm culture dishes. Hams F12 medium supplemented with 10% FBS, 20 mM HEPES, 100 U/ml  
10 penicillin, 100 µg streptomycin, and 2 mM L-glutamine was used to maintain the cell lines prior to the procedure. More particularly, the TRVB-1 line, which does not express the hamster TR but expresses the transfected human TR, was maintained in the same media with the addition of 100µg/ml  
15 G418 sulfate (Gibco). The cells were incubated at 37°C in a humidified 5% CO<sub>2</sub> environment until they were 80% to 85% confluent.

Mixed DNA (27µg pSV2p97a (ATCC No. CRL 9304) in 3 µl, 0.5 µg pWJ218 (see Figure 3) in 0.5 µl, and 46.5 µl  
20 sterile distilled water) was combined with 50 µl of Lipofectin<sup>®</sup> Reagent (Life Technologies Inc./Bethesda Research Laboratories, Gaithersburg MD) according to the manufacturers' instructions. The cells were then washed twice with 3 ml of serum-free Hams F12 medium, resuspended  
25 in 3.0 ml of medium and gently swirled in tissue culture dishes. More particularly, the plasmids pSV2 p97a, a human p97 expression vector containing the entire coding region of p97 cDNA driven by the SV40 early promoter (ATCC NO. 9304), and pWJ218 containing the G1418 resistance gene  
30 were cotransfected into the cell lines by the Lipofectin<sup>®</sup> method (Gibco, New York) following the procedure recommended by the manufacturer. The cells were then incubated for 36 hours at 37°C in a humidified, 5% CO<sub>2</sub> environment.

35       An equal volume of Hams F12 medium containing 20% Fetal Bovine Serum (FBS) and 1600 µg/ml G418

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(Geneticin/Gibco) was added to the tissue culture dishes. The cells were washed and the media (Hams F12 with all supplements including 10% FBS and 800 µg/ml G418) changed daily for a week. Utilizing the anti-p97 antibody L235 (more particularly, L235 is an IgG monoclonal antibody secreted by the hybridoma cell line ATCC No. HB 8446) cell populations expressing p97 were analyzed by flow cytometry ("FACS"). Positive cell populations were then further sorted for cells which expressed higher levels of p97.

More particularly, the cells were counted ( $10^6$  cells/tube) and washed twice in fluorescence activated cell sorting ("FACS") buffer, which consisted of DMEM containing 0.5% (wt.vol) bovine serum albumin, 20 mM HEPES, and 20 mM  $\text{NaN}_3$ . The cells were incubated with the various monoclonal antibodies for 45 min at 4°C, then washed and labelled with the appropriate fluoresceinated secondary antibody for 45 min at 4°C. The cells were then washed and fixed in 1.5% (vol/vol) p-formaldehyde in PBS. A Becton-Dickinson FACScan flow cytometer was used to measure 5000 events per sample. The fluorescence intensities were normalized with respect to unstained control samples. The following primary antibodies were used in immunohistochemistry studies: anti-human MTF(L235, 1:1,000 dilution, mouse monoclonal, IgG<sub>1</sub>, ATCC (HB104); anti-human Tf(A-061, 1:1,000 dilution, rabbit polyclonal, DAKO); and anti-human TR (OKT9, 1:1,000 dilution, mouse monoclonal, IgG<sub>1</sub>, ATCC CRL 8021). Tissue culture supernatants or Protein G column (Pharmacia) purified preparations were used as a source of antibody in the experiments. Positive cell populations were then further sorted for cells which expressed higher levels of p97.

Positive cells were then sub-cloned by limiting dilution. The resultant cell lines were once again analyzed by FACS to ensure high expression of p97. Two clones which stably expressed high levels of p97 were isolated: p97aWTBc3 and p97aWTBc7.

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EXAMPLE 2**EXPRESSION OF P97 ON THE SURFACE OF CELLS  
TRANSFECTED WITH p97 cDNA****A. Preparation of Plasmid D5-9(+)**

5       The expression vector pVL1393 (see, Luckow, "Cloning and Expression of Heterologous Genes in Insect Cells with Baculovirus Vectors", Cloning Techniques and Applications, pp. 122-123) was digested with SmaI, followed by digestion with Calf Intestinal Phosphatase ("CIP") to prevent self-  
10   ligation.

Human p97 cDNA from plasmid pSV2p97a was amplified, and a miniprep of plasmid DNA prepared. Plasmid DNA was then digested with HindIII and NruI, and a 233 bp fragment isolated from a 1% agarose gel in TAE. The 3' overhang  
15   created by the HindIII digest was filled in, and the fragment was purified.

The HindIII-NruI p97 fragment and the pVL1393/SmaI CIP linearized vector were ligated, and used to transform competent DH5 $\alpha$  cells (Hanahan, D., DNA Cloning Vol 1, A  
20   Practical Approach Series, Glover, ed., Chapter 6, pp. 10-135, IRL press, 1985). Positive clones were picked, and plasmid DNA was produced from minipreps of each clone. A particularly preferred plasmid, D5-9(+), is schematically depicted in Figure 4.

**25   B. Transfection of Sf9 Cells**

Spodoptera frugiperda or "Sf9" cells (ATCC No. CRL 1711) were transfected with a mixture of wild type AcMNPV genomic DNA and D5-9(+) plasmid DNA described above, essentially according to the method of Summers and Smith  
30   (A Manual of Methods for Baculovirus Vectors and Insect Cell Culture Procedures, Texas Agricultural Experiment Station, Bulletin No. 1555, 1988 (1988; Section 4.4.1 Transfection of Sf9 Cells - Method I), in order to incorporate the human p97 gene into the AcMNPV genome.

35       Human p97 recombinant viruses were purified using a



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plaque assay described by Summers and Smith, supra. Three rounds of plaque assays were done in order to isolate the recombinant viruses carrying the p97 gene. These included: Round 1:  $10^{-5}$ , dilutions of transfection mix; 5 Round 2:  $10^{-1}$ ,  $10^{-2}$ , dilutions of plaques picked in round 1; and Round 3:  $10^{-3}$ ,  $10^{-4}$ , dilutions of plaques picked in round 1.

### C. Results

SK-MEL-28 cells (which are known to express p97 and, 10 for greater clarity, are a human melanoma cell line, ATCC HTB72), uninfected Sf9 cells, wild type AcMNPV infected Sf9 cells, and p97 recombinant virus infected Sf9 (p97 B-1-1 and p97 B-2-1) were analyzed by Fluorescence Activated Cell Sorting (FACS) to detect the expression of p97 on the 15 cell surface. Hybridoma supernatant from anti-p97 antibody L235 was used as the first antibody and goat anti-mouse (GAM) IgG-FlTC antibodies were used as the second antibody. Controls were treated with No Fluorescent Antibody ("NFA"), and PI-PLC (which cleaves the GPI 20 anchor, releasing p97 from the cell surface).

As can be seen from Table I below, SK-MEL-28 cells and the p97 recombinant virus infected Sf9 cells (p97 B-1-1 and p97 B-2-1) were positive for p97 expression while the uninfected Sf9 cells and the wild type virus infected 25 Sf9 cells were not. In addition, when the samples were pre-incubated at 37°C for 60 minutes with PI-PLC and then labelled with first and second antibodies, the amount of p97 on the surface of SK-MEL-28 cells, and on the surface of the p97 recombinant virus infected Sf9 cells was 30 reduced drastically. This result suggests that p97 expressed on the surface of Sf9 cells is attached by a lipid anchor as it is in SK-MEL-28 cells.

### EXAMPLE 3

#### RELEASE OF P97 BY BACTERIAL PI-PLC IN TRANSFECTED CELL LINES

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#### A. Effect of PI-PLC on Cells

PI-PLC was prepared by first transfecting a culture of *Bacillus subtilis* (BG2320) with the PI-PLC gene from *Bacillus thuringiensis*. PI-PLC was then purified from the supernatants of transfected cells essentially according to the procedure described by Low et al. in *J. Immunol. Methods* 113:101-111, 1988.

More particularly, *B. Subtilis* (BG2320) transfected with the gene for PI-PLC from *B. thuringiensis* (Henner et al., *Nucleic Acids Research* 16:10383, 1988) was cultured using a procedure adapted from that previously used to grow *B. thuringiensis* (Low et al., *J. Immunol. Methods* 113:101-111, 1988). The growth medium containing 10 g/L Polypeptone, 10 g/L yeast extract, 5 g/L NaCl, 0.4 g/L  $\text{Na}_2\text{HPO}_4$  and 15  $\mu\text{g/ml}$  chloramphenicol (pH adjusted to 7.0 with NaOH) was inoculated with 1.5-3% (v/v) of overnight preculture (initial).  $D_{600}=0.1$ ). Cells were cultured in Erlenmeyer flasks and shaken at 150 rmp, 37°C for 6 to 12 hours. Cells were removed by centrifugation and the supernatant filtered through a 0.2  $\mu\text{m}$  membrane (VacuCap, Gelman Sciences, MI). The supernatant was concentrated 20-fold using an ultrafiltration cell (Model 8400, Amicon Corp. MA) and a 10,000 MW YM10 ultrafilter (Amicon, MA). The concentrated enzyme solution was then washed two times with 5 volumes of PBS in the ultrafiltration cell. The enzyme solution was assayed and stored in 1 ml aliquots at - 20°C. When the enzyme was required, the frozen PI-PLC was rapidly thawed and diluted in PBS to the specified concentrations. All enzyme samples used in this study came from the same 2L batch fermentation.

SK-MEL-28 (ATCC No. HTB 72) cells and p97aWTBc3 cells (prepared as described above) were grown up, counted, placed into tubes ( $10^6$  cells/tube), and washed two times with FACS buffer (DMEM containing 0.5% (wt/vol) bovine serum albumin, 20 mM HEPES, and 20 mM  $\text{NaN}_3$ ). The cells were then incubated for 1 hour at 37°C with purified phosphatidylinositol-specific phospholipase C (PI-PLC) at

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a concentration of  $1.7 \text{ U}/10^6$  cells in FACS buffer. The cells were washed, and stained with anti-p97 antibody L235 at  $4^\circ\text{C}$  for 45 min. The cells were washed again in FACS buffer and then incubated with fluoresceinated goat anti-mouse IgG at  $4^\circ\text{C}$  for 45 min. The cells were then washed and fixed in 1.5% (vol/vol) p-formaldehyde in PBS.

A Becton-Dickinson FACScan flow cytometer was used to measure 5000 events per sample. More particularly, data from individual experiments were compared to unstained negative controls and values expressed as percentages of untreated positive controls. The results are set forth in Figure 5. Briefly, as shown in Figures 5(c) and (d), a significantly higher fluorescent intensity resulted for both SK-MEL-28 cells and p97aWTBc3 cells which were not treated with PI-PLC as compared to those that were (Figures 5e and f).

Control cells which were not stained with anti-p97 antibodies (Figures 5a and 5b), showed only background fluorescence. Therefore, the treatment of the SK-MEL-28 and p97a WTBc3 cell with bacterial PI-PLC resulted in a decrease in p97 expression at the cell surface.

#### B. Pronase Treatment of Cells

In order to determine whether the bacterial PI-PLC contained a non-specific protease, Pronase was used to treat cells which were then stained for either p97 or the transferrin receptor. Briefly, cells were treated as described above, except that cells were incubated with 1 mg/ml of Pronase (a Type XIV Protease from *Streptomyces griseus* (Sigma Chemical Co.)) for 1 hour in FACS buffer at  $37^\circ\text{C}$ , rather than with PI-PLC.

Also included in this study were EL-4 cells which express the Thy-1 and transferrin receptor proteins on its cell surface. For greater clarity, EL-4 cells are mouse lymphoma cells (ATCC TIB 39). Thy-1 is known to be GPI-anchored to the cell surface.

Results are presented in Table 2 below for SK-MEL-28,

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EL-4, p97aWTBc3 and p97aWTBc7 cells. For these experiments, p97 was labelled with monoclonal antibody L235 and the transferrin receptor was labelled with monoclonal OKT9. Thy-1 was labelled with the T24/37.1 MAB  
5 (obtained from Dr. R. Hyman, The Salk Institute, San Diego, CA) while the mouse transferrin receptor was labelled with the monoclonal antibody  $\lambda$ E1/9.9.3 (obtained from Dr. F. Takei, the University of British Columbia, Canada). Appropriate fluoresceinated secondary antibodies  
10 were used according to the type of primary antibody. The results were converted from logarithmic to linear scale using the formula: linear mean fluorescence =  $10^{(\log \text{ mean fluorescence}/256 \text{ channels})}$ . Fluorescence intensities were normalized with respect to unstained control samples and values  
15 expressed as percentages ( $\pm$  s.d.) of untreated stained control samples.

As shown in Table 2 below, both the p97 protein on SK-MEL-28 cells and the Thy-1 protein on EL-4 cells were sensitive to the effects of PI-PLC, but not to the effects  
20 of Pronase. In contrast, the transferrin receptors on both SK-MEL-28 cells and EL-4 cells were sensitive to Pronase, but not PI-PLC.

This data also shows that the amount of p97 expression on SK-MEL-28 cells was decreased to 10% of  
25 initial levels by treatment with bacterial PI-PLC. In contrast, the expression of the human transferrin receptor (TR) on SK-MEL-28 cells was not changed at all by bacterial PI-PLC treatment.

#### EXAMPLE 4

##### 30 AFFINITY PURIFICATION OF p97

##### A. Preparation of the Affinity Matrix

p97 was affinity purified essentially as described below. Briefly, 2 ml of mixed beads (Protein A Sepharose CL-4B, Pharmacia #17-0963-03) were removed from the vial  
35 and washed three times in 5 ml of 0.1 M borate (pH 8.2). The beads were then resuspended in 6 ml of borate (pH 8.2)

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containing 2.5 mg of Rabbit Anti-Mouse IgG (Jackson Immunoresearch, #315-005-003), and incubated for 60 minutes at 4°C with shaking. The beads were then centrifuged at 1800 rpm for 3 minutes, followed by three cycles of washing with 5 ml 0.1 M borate (pH 8.2).

L35 hybridoma supernatant (mouse anti-human p97 IgG) was added to the beads and incubated for 60 minutes at 4°C with shaking. The beads were then centrifuged as described above and washed three times in 5 ml 0.1 M borate (pH 8.2), followed by washing three times in 5 ml 0.2 M triethanolamine (pH 8.2). The beads were then resuspended in 20 ml Dimethyl Pimelidate HCl in 0.2 M triethanolamine, and thereafter centrifuged (500 x g) for 1 minute. The cells were resuspended in 20 ml of 20 mM ethanolamine (pH 8.2), and incubated for 5 minutes at 22°C. The beads were then washed three times with 5 ml 0.1 M borate (pH 8.2), and stored at 4°C in 5 ml of 0.1 M borate containing 20 mM azide.

#### B. Purification of p97 on the Affinity Column

p97aTRV6c3 cells were grown to 75-95% confluence in Hams medium supplemented with LBS, HEPES, L-Glu, FEMS, and 800µg G418. 100ml of the medium was concentrated with an Amicon Centriprep 30 and purified using affinity chromatography as described below. An affinity column for the purification of p97 was prepared essentially as described in J. Biol. Chem. 257:10766-10769, 1982. Briefly, beads (prepared as described above) were washed with 0.1 M borate buffer (10 ml), and utilized to prepare the affinity column. The column was then pre-eluted with 2 ml of elution buffer 0.05 diethylamine, pH 11.5, containing 0.5% sodium deoxycholate and a sample of tissue culture media from cells were then passed through the column. The column was then washed successively with 5 ml of buffer B (0.2% NP-40, 150 mM NaCl, 2 mM EDTA, 10 mM Tris-HCl pH 7.5), 5 ml of buffer C (0.2% NP-40, 0.5 M NaCl, 2 mM EDTA, 10 mM Tris-HCl pH 7.5) and 5 ml of buffer

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D (10 mM Tris-HCl pH 7.5). Five milliliters of elution buffer was then added, and 1 ml aliquots were collected. One hundred microliters of 0.5 M  $\text{NaH}_2\text{PO}_4$  was added to each of the aliquots in order to bring the sample to  
5 neutrality.

Purity was tested by PAGE (with Coomassie blue, silver staining, or autoradiography), and spectrophotometry. Figure 34 shows the purification of p-97.

10

#### EXAMPLE 5

#### **CELL SURFACE BIOTINYLATION, PI-PLC TREATMENT, AND IMMUNOPRECIPITATION**

The effect of bacterial PI-PLC and the specificity of monoclonal antibody L235 was further characterized in  
15 Figure 6 where surface proteins from either WTB (lane 1), p97aWTBc7 (lane 2) or SK-MEL-28 cells (lane 3) were labelled with Biotin, followed by immunoprecipitation of p97 and analysis by SDS-PAGE under reducing conditions.

Briefly, surface proteins of  $3.0 \times 10^6$  SK-MEL-28 cells  
20 were labelled with 0.2 mg Biotinamidocaproate N-Hydroxysuccinimide Ester (Biotin, Sigma) essentially as described by von Boxberg et al. (Eur. J. Biochem. 190:249-256, 1990). The cells were washed several times in DMEM, and divided into two samples that were incubated for 60  
25 min at 6°C in the presence or absence of PI-PLC ( $1.7 \text{ U}/10^6$  cells) respectively. Both the cell supernatant and the cell pellet were subsequently processed. The cells were washed once more and lysed in 50 mM Tris-HCl pH 7.4, 150 mM NaCl, 2mM EDTA, 0.5% NP-40, 1mM  
30 phenylmethylsulfonylfluoride (PMSF) and 100 µg/ml lysine to block the excess of free Biotin. The same buffer was added to the supernatant. The samples were centrifuged at 12,000 g for 10 min at 4°C to remove the cell nuclei and cell debris. The samples were precleared for 2 h with  
35 washed protein A-agarose.

The p97 was immunoprecipitated with antibody L235

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followed by protein A-agarose precoated with rabbit anti-mouse IgG (Jackson ImmunoResearch). After immunoprecipitation, the beads were washed 6 times in 50 mM Tris-HCl pH 6.5, 150 mM NaCl, 2mM EDTA, and 0.5% NP-40.

5 The proteins were eluted from the beads in sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis (PAGE) loading buffer and separated on an 8% SDS-PAGE gel under reducing conditions. The proteins were transferred onto Immobilon membranes (Millipore) by electroblotting, and  
10 detected using peroxidase-conjugated streptavidin (Jackson ImmunoResearch) and the chemiluminescence ECL Western blotting detection system (Amersham) using the conditions recommended by the manufacturer.

As shown in Figure 6, a single protein of 95-97,000  
15 daltons molecular mass was immunoprecipitated in both lane 2 (p97aWTBc7) and in smaller quantities in lane 3 (SK-MEL-28), but not from lane 1 (WTB). PI-PLC treatment of the cells resulted in a decreased amount of protein due to a large loss of protein from the cell surface (compare  
20 Figure 6A, lanes 2 (+) and (-)), which was subsequently recovered in the cell supernatant (Figure 6B). Under the conditions used in this experiment, no difference in the molecular mass between the plasma membrane associated form and the released form could be detected.

25

#### EXAMPLE 6

##### **BIOSYNTHETIC LABELLING WITH [<sup>3</sup>H]-ETHANOLAMINE**

In order to determine whether the decrease in expression of p97 observed after bacterial PI-PLC treatment was an indirect effect due to the association of  
30 p97 with another PI-PLC sensitive protein at the cell surface, SK-MEL-28 cells were biosynthetically labelled with [<sup>3</sup>H] ethanolamine, which is known to be a component of the phospholipid moiety of GPI-anchored proteins.

Briefly, SK-MEL-28 cell line monolayers were  
35 biosynthetically labelled for 24 hours with [<sup>3</sup>H] ethanol-1-ol-2-amine hydrochloride (20 µCi/ml, 30.4 Ci/mmol,

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Amersham) in DMEM containing 5% dialyzed FBS, 20 mM HEPES, 100 U/ml penicillin, 100 µg/ml streptomycin, 2 mM L-glutamine, and  $5.0 \times 10^{-5}$  M 2-mercaptoethanol. The cells were washed in PBS and lysed in 20 mM Tris-HCl pH 7.2, 150 mM NaCl, 2 mM EDTA and 0.5% NP-40 with 20 µg/ml PMSF. The lysates and cell supernatants were then cleared by centrifugation, in particular at 100,000 g for 1 hour, prior to the immunoprecipitation. The primary antibodies used were the L235 for p97 and the OKT9 for the human TR. Protein A-agarose (BioRad) coated with rabbit anti-mouse IgG antibody (Jackson ImmunoResearch) was added to the samples and incubated for 8 hours at 4°C. The resulting complex was washed in 50mM Tris HCl pH 6.5, 150mM NaCl, 2mM EDTA, and 0.5% NP-40 and resuspended into SDS-PAGE loading buffer. The samples were run under reducing conditions on a 10-15% gradient SDS-PAGE gel. After fixation the gel was treated with Amplify™ (Amersham), dried, and autoradiographed.

As shown in Figure 7, in Lanes 1 and 2 p97 was immunoprecipitated with the anti-p97 antibody L235, and is visible due to labelling by [ $^3$ H]-ethanolamine. In contrast, the human transferrin receptor was immunoprecipitated with the anti-transferrin receptor antibody OKT9 in lanes 3 and 4, but is not visible because it was not labelled by the [ $^3$ H]-ethanolamine.

WTB cells (Lanes 1,2) and p97aWTBc3 cells (Lanes 3,4) were biosynthetically labelled with [ $^3$ H]-ethanolamine following the procedures described above. Proteins were then precipitated by the anti-p97 antibody L235 (Lanes 1 and 3), and the anti-transferrin receptor antibody (Lanes 2 and 4). As is shown in Figure 8, only protein (p97) from the p97aWTBc3 cell line precipitated and was labelled with [ $^3$ H]-ethanolamine.

#### EXAMPLE 7

#### PHASE SEPARATION OF p97 IN TRITON X-114

The technique of phase separation in Triton X-114 can



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be used to assess the amphipathic or hydrophilic character of a protein and is especially useful to identify GPI-anchored proteins. This technique is based on the ability of the detergent Triton X-114 to partition into two phases: a detergent rich phase and a detergent poor phase. Amphipathic proteins which possess a hydrophobic membrane anchor, such as a GPI anchor, partition into the detergent rich phase, whereas hydrophilic proteins partition into the aqueous phase.

10 In order to investigate p97 partitioning in Triton X-114, the cell surface proteins of  $8.0 \times 10^6$  SK-MEL-28 cells were labelled with 0.4 mg Biotin (Sigma) as described above, following the methods described in von Borberg, Y. et al., (Eur. J. Biochem., supra). The cells were washed  
15 several times in DMEM, divided into two samples that were incubated for 60 min at 6°C, in the presence or absence of PI-PLC ( $1.7 \text{ U}/10^6$  cells) respectively. Both the cell supernatant and the cell pellet were subsequently processed. The cells were washed once more and lysed in a  
20 buffer containing 10 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% Triton X-114, 1mM PMSF and 100 µg/ml lysine to block Biotin. Triton X-114 (Sigma) was precondensated as described by Bordier (J. Biol. Chem. 256:1604-1607, 1981). The same buffer was added to the supernatant. The samples  
25 were centrifuged at 12,000 g for 10 min at 4°C to remove the cell nuclei and cell debris. The phase separation was obtained by incubation at 30°C followed by a centrifugation at 3000 g for 3 min at room temperature. The samples were re-extracted 3 times in order to improve  
30 the separation and the corresponding phases were pooled.

The samples were precleared for 2 hours with washed protein A-agarose and subsequently divided into two halves for immunoprecipitation of p97 and the transferrin receptor using L235 and OKT9 monoclonal antibodies,  
35 respectively, followed by protein A-agarose precoated with rabbit anti-mouse IgG (Jackson ImmunoResearch). After immunoprecipitation, the samples were washed 6 times in 50

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mM Tris-HCl pH 6.5, 150 mM NaCl, 2 mM EDTA, and 0.5% NP-40. The proteins were eluted from beads in SDS-PAGE loading buffer and separated on an 8% SDS-PAGE gel under reducing conditions. The proteins were transferred onto  
5 Immobilon membranes (Millipore) by electroblotting, and detected using peroxidase-conjugated streptavidin (Jackson ImmunoResearch) and the chemiluminescence ECL Western blotting detection system (Amersham) using the conditions recommended by the manufacturers.

10        Figure 9 depicts results from cells which were washed in DMEM and incubated in the presence (+) or absence (-) of PI-PLC (1.7 U/10<sup>6</sup> cells) for 60 min at 6°C. Proteins from the cell pellet (P) or the cell supernatant (S) were separated in Triton X-114 solution, and p97 and TR were  
15 immunoprecipitated from both the aqueous phase (A) or the detergent phase (D). Figure 9 shows that all p97 molecules expressed at the surface of untreated human melanoma SK-MEL-28 cells partition into the detergent-rich phase. No p97 was detected in the supernatants of  
20 untreated cells. Treatment with bacterial PI-PLC led to the partitioning of p97 into the aqueous phase of the cell supernatant sample, indicating that the protein was cleaved from the plasma membrane and released as a hydrophilic form. No p97 could be detected in the  
25 bacterial PI-PLC treated cell pellet, indicating that most molecules were bacterial PI-PLC sensitive and that p97 is not simultaneously expressed in a transmembrane and GPI-anchored form at the cell surface. In contrast to p97, the TR, which is inserted in the membrane through a  
30 hydrophobic peptide segment, is not affected by bacterial PI-PLC. The amphiphilic structure causes the protein to partition in both phases after separation.

#### EXAMPLE 8

SPECIFICITY OF THE ANTI-P97 ANTIBODY L235, AND THE  
ANTI-TRANSFERRIN RECEPTOR ANTIBODY OKT9

35

##### A. Cell Surface Expression

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The reactivities of the anti-p97 antibody L235 and OKT9 were confirmed. More particularly, the cell surface expression of human p97 and human TR were tested by staining the SK-MEL-28 cells with the L235 and OKT9 MAb and analyzing by FACS as outlined in Example 1. The human p97 molecule was shown to be expressed at a greater level than human TR. The expression of p97 by the p97aWTBc3 cell line (See Example 1 re preparation of p97aWTBc3) was found to be considerably higher than the SK-MEL-28 cell line (Figure 10). The specificity of the L235 MAb to p97 was confirmed by the lack of reactivity to the parental (untransfected) CHO cell line WTB. At the same time the specificity of the OKT9 MAb for the human TR was demonstrated. The reactivity which is evident in the SK-MEL-28 line is absent in the WTB and p97aWTBc3 lines but is present in the TRVb-1 line.

#### B. Biosynthetic Labelling

The fates of p97 and TR after biosynthetic labeling of SK-MEL-28, WTB and p97aWTBc3 cells were also examined. SK-MEL-28, WTB and the p97 transfected p97aWTBc3 cells were cultured on petri dishes until reaching 80-90% confluence. The cells were then treated in minimal medium lacking methionine for 1 hour prior to labeling. Biosynthetic labeling of cells was done during 15 minutes with 2 ml of 150 uCi/ml of [<sup>35</sup>S]-methionine per petri dish. Cells were then chased with normal medium containing an excess of cold methionine for various times. A separate petri dish was used for each time point. The cells were lysed in 20mM Tris-HCl pH 7.2, 150 mM NaCl, 2mM EDTA, and 1% NP-40 with 20 ug/ml PMSF. The lysates and cell supernatants were then cleared by centrifugation prior to immunoprecipitation. The primary antibodies used were L235 and OKT9 as described above. Immunoprecipitation and SDS-PAGE analysis were carried out as described in Kvist et al. (1982, Cell 29, 61-69).

The L235 MAb recognized a protein with a mw of 93 kDa

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(Figure 11, lane 1) that is processed to a higher mw of 95 kDa after 6 h of chase (Figure 11, lanes 1, 2). This protein was not seen in WTB cells (Figure 11, lanes 1, 2). A considerably greater amount of this protein is present in the p97 transfected CHO cells, p97aWTBc3 (Figure 11, lanes 1, 2) identifying this protein as p97. In addition, a secreted form of p97 is present in the cell supernatant after 6 h of chase (Figure 11, lane 4). The OKT9 MAb recognizes a protein with similar molecular weight in SK-MEL-28 cells corresponding to the reduced form of the human TR (Figure 11, lanes 5, 6). The human TR is not seen in the cell supernatant (Figure 11, lanes 7, 8). It is clear that the L235 and OKT9 MAb do not cross react with hamster p97 and TR in the CHO line WTB.

This data separately and collectively confirm the specificity of the L235 antibody for p97. They also confirm that p97 and TR are synthesized and transported to the cell surface. A form of p97 was also identified in the medium.

20

#### EXAMPLE 9

##### **BIOSYNTHESIS AND TRANSPORT OF P97**

##### **A. [<sup>35</sup>S]-Methionine Pulse-Chase Experiments**

In order to investigate the biosynthesis and transport of p97 in melanoma cells, pulse-chase experiments were performed. Briefly, SK-MEL-28 cells were metabolically labeled with 150  $\mu$ Ci/ml of [<sup>35</sup>S]-methionine for 15 minutes, washed and subsequently chased with normal medium containing an excess of cold methionine at various timepoints. At each time point the supernatants from the cell cultures were collected in a separate petri dish, and the cells were lysed in nonionic detergent (20 mM Tris-HCl pH 7.2, 50 mM NaCl, 2 mM EDTA and 1% NP-40 with 20  $\mu$ g/ml PM:F). The lysates and cell supernatants were then cleared by centrifugation (100,000 for 1 h) prior to the immune precipitation. The primary antibodies used were L235 which recognizes p97 and OKT9 which recognizes

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the human transferrin receptor. The p97 molecule and, as a control, the TR, were immunoprecipitated from both the cell lysate (Figure 12, lanes 1-7) and from the tissue culture supernatant (Figure 12, lanes 8-14) and analyzed by SDS-PAGE.

As shown in Figure 12, the p97 molecule is processed to a higher molecular weight form during the chase (Figure 12, lanes 1-7). The processing of p97 is up to four times slower than the processing of the TR (Figure 12, lanes 1-7). In addition, p97 is secreted into the medium (Figure 12, lanes 8-14), whereas no TR is found in the medium. The appearance of the secreted form can be detected after only 1 hour of chase on an overexposed gel, indicating a transport rate of the secreted form that is comparable to the membrane associated form.

**B. Endo H Digestion During [<sup>35</sup>S]-Methionine Pulse-Chase**

The transport of glycoproteins can be assessed by the modification of their glycans during successive exposure to Golgi specific enzymes becoming resistant to Endoglycosidase H (Endo H) digestion. Briefly, SK-MEL-28 cells were labeled for 15 min with [<sup>35</sup>S]-methionine, and chased with an excess of unlabeled methionine for the time indicated at the bottom of Figure 13, lysed, and subjected to immunoprecipitation with L235 MAb (p97) and OKT9 MAB (TR) as described above. Precipitates were digested with 5 mM of Endo H (Boehringer Mannheim) for 20 h at 37°C, and analysed as described above. The autoradiograms were developed after 3 days exposure of the gel.

As shown in Figure 13, most of the p97 molecules are Endo H resistant after 4 hour chase (Figure 13, lane 6), in comparison, only 1 hour chase is necessary for the TR to become resistant to Endo H digestion (Figure 13, lane 4). It therefore appears that the transport rate of both the secreted and GPI-anchored forms of p97 is much slower than the transport rate of TR. This difference may indicate that p97 needs more time in order to achieve a conformation or a structure allowing transport through the

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Golgi and to the cell surface. Also, the secreted form of p97 is resistant to Endo H digestion, indicating that the soluble form uses the normal secretory pathway for transport to the cell surface. Briefly, SK-MEL-28 cells were pulse labelled for 15 minutes with 200  $\mu$ Ci/ml  $^{35}$ S-methionine and chased in medium with excess cold methionine for 0 minutes, 30 minutes, 1, 2, 4, 8 or 24 hours. Proteins were immunoprecipitated using L235 and OKT-9 antibodies, using protein A sepharose precoated with rabbit anti-mouse IgG. Prior to pulse-chase labelling, cells were pre-treated with minimum medium without methionine. Radioactive cell lysates and cell supernatants were precleared with normal rabbit serum before immunoprecipitation. The results are shown in Figure 35. The upper band corresponds to the soluble form and is resistant to Endo H digestion.

C. Triton X-114 Phase Separation of [ $^{35}$ S]-Methionine Labeled SK-MEL-28 Cells

The secreted form of p97 was analyzed by Triton X-114 phase separation on the cell supernatant (Figure 14). Briefly, SK-MEL-28 cells were labeled for 30 min with [ $^{35}$ S]-methionine and chased with an excess of cold methionine for the time indicated on the top of Figure 14. The aqueous (A) and detergent (D) phases from the medium were analysed after Triton X-114 phase separation, immunoprecipitated with L235 MAb (p97) and run on SDS-PAGE as described above. The autoradiogram was developed after 4 days exposure to the gel.

As shown in Figure 14, p97 secreted in the cell medium after a 4 hour-pulse and a 24 hour-chase partitioned in the aqueous phase, demonstrating that this form has no hydrophobic tail. In addition, after 24 hours labeling with [ $^3$ H]-ethanolamine, and 30 days exposure, ethanolamine labeled p97 could not be detected in medium from p97 transfected p97aWTBc3 cells (Figure 15, lanes 3 and 4) and SK-MEL-28 cells (Figure 15, lanes 7 and 8). It

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is clear that there is [<sup>3</sup>H]-ethanolamine labeled p97 (Figure 15, lanes 1, 2 and 6) associated with the detergent lysates of the p97aWTBc3 and SK-MEL-28 cells, but the secreted form of p97 is not labeled. In a similar  
5 experiment with [<sup>35</sup>S]-methionine labeling, the secreted form is clearly evident in a 3-day exposure of a 15 minute labeling (Figure 12).

In order to determine whether the secreted form of p97 originates from either the release of cell surface  
10 GPI-anchored p97 or from a synthesized soluble form that is secreted into the medium, the fate of cell surface biotinylated p97 was followed (Figure 16). Cell surface biotinylated p97 and TR were chased in normal medium, and analyzed by SDS-PAGE after immunoprecipitation,  
15 electroblotting, and detection by peroxidase conjugated streptavidin and the chemiluminescence ECL Western blotting detection system. The aqueous and detergent phase of Triton X-114 from cell lysate and medium were then analyzed.

20 As shown in Figure 16, no p97 is accumulated in the medium after 6 hour chase (Figure 16, lanes 11 and 12). p97, like TR, was always associated with cells (Figure 16, lanes 1, 2, 5, 6, 9 and 10) and always partitioned in the detergent phase indicative of a hydrophobic protein  
25 (Figure 16, lanes 2, 6, and 10). It therefore appears that two forms of p97 are synthesized, one is membrane bound by GPI-anchor and remains on the cell surface, and a second form is secreted into the medium.

#### EXAMPLE 10

##### 30 LOCALIZATION OF P97 IN BRAIN SECTIONS BY INDIRECT IMMUNOPEROXIDASE

##### A. Expression of the transferrin receptor and p97 in healthy and Alzheimer's Disease brain tissues

Thirty brains were examined, including 7 Alzheimer's  
35 disease (AD) (aged 67-81), 5 Parkinson's disease (PD) (aged 69-76) (3 of them had AD changes), 3 progressive

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supranuclear palsy (PSP) (60-66), 3 Huntington's chorea (HD) (aged 49-63), 2 multiple sclerosis (MS) (aged 56-66), 4 amyotrophic lateral sclerosis (ALS) (aged 48-81) and 7 non-neurological controls (aged 54- 82). Brains in all cases were obtained 2-32 hours after death. Briefly, small blocks were dissected from various brain regions of non-neurological cases, angular, entorhinal cortices and hippocampus of AD, angular entorhinal cortices, hippocampus and substantia nigra in PD, precentral cortex, basal ganglia of PSP, striatum of HD, cerebral white matter having plaques of MS and precentral gyrus and spinal cord of ALS. These blocks were fixed for two days in phosphate-buffered 4% paraformaldehyde. They were then transferred to a maintenance solution of 15% sucrose in 0.1M phosphate buffer, pH 7.4, and kept in the cold until used. Sections were cut on a freezing microtome at 30 mm thickness and stained by single or double immunohistochemical procedures (McGeer et al., 1992) using primary antibodies. The antibodies and their dilutions were: anti-human p97, 1:1000 (murine monoclonal L235, American Type Cell Culture HB 8446; R-17, 1:10,000 (rabbit polyclonal against BAP, generously provided by Dr. Ishii); anti-HLA-DR antibody, 1:1,000; anti-human transferrin receptor antibody OKT9, American Type Cell Culture CRL 8021). For greater clarity, the specificities of the L235 monoclonal antibody and the OKT9 MAb monoclonal antibody for p97 and TR respectively were confirmed as set forth herein.

**B. Expression of p97 and TR in Healthy and AD Brain Tissues**

Immunohistology methods were used to establish the distribution of p97 and TR in healthy human brain. The staining of control human brain tissue with the L235 (Figure 17a), A-061 (Figure 17b), and OKT9 MAb (Figure 17c) is shown. In control human brain tissue (cortex), capillary endothelium were strongly stained by L235



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(Figure 17a) and OKT9 (Figure 17c). In contrast the pattern of staining which was obtained with the anti-transferrin MAb is completely different and is not localised to these structures (Figure 17e).

5       The limited staining with A-061 MAb is localised to small round oligodendrocytes as previously proposed (Connor et al., 1990). These data establish the coincident expression of TR and p97 on capillary endothelium in normal brain which form the blood/brain  
10 barrier and the Tf is not found in association with TR or p97. These results are suggestive of a close association between the functions of p97 and TR.

      The identical pattern of expression of both TR and p97 was further investigated in neuropathological brain  
15 tissue. A comparison of normal and AD brain tissues stained for MTf and TR again demonstrated the coincident expression of p97 and TR.

      Figure 17A shows that normal angular cortical gray matter stained with anti-p97 MAb. Only capillary  
20 endothelium is positive. Figure 17B indicates that angular cortical gray matter stained with anti-p97 MAb and, in addition, some microglia are positively stained (arrows). Figure 17C shows a section nearby to section 17A stained with anti-transferrin receptor MAb. As in  
25 Figure 17A, only capillary endothelium is positive. Figure 17D shows a nearby section to B, stained with anti-transferrin receptor MAb. As in B, capillaries and some microglia (arrows) are positive. Figure 17E shows normal angular cortex stained with anti-transferrin polyclonal  
30 antibody. The sparse cytoplasm of a few cells resembling oligodendrocytes (arrows) are positive. Figure 17F shows angular cortex from Alzheimer's Disease brain stained with anti-transferrin polyclonal antibody. Sparse cells resembling oligodendrocytes (arrows) and rare cells  
35 resembling microglia (arrow) are positive.

      The binding of the OKT9 antibody to capillary endothelial cells in Alzheimer's Disease brains is

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identical to that seen in control brains but, in addition, a subset of microglial cells is also stained. By contrast, the anti-transferrin MAb failed to stain capillaries, and stained only occasional oligodendrocytes and microglia in the white matter (Figure 17F). Staining of sections of AD and normal tissue from the same region of the brain cortex, with the L235 MAb revealed that the distribution of p97 is identical to that of the TR (Figure 17c, 17d). Both microglial cells and endothelial cells are labelled. In experiments on other pathologically affected brain tissue from cases of PD, PSP, HD, MS or ALS, no microglial labelling was seen with either the L235 or OKT9 MAb. In control experiments in which the primary antibody was omitted, no labelling of cells or any other structures was seen in AD or control brain tissue. These data support the unique and identical distributions of p97 and TR in AD tissue.

Upon viewing of the brain sections noted above, it was evident that the labelling of Alzheimer's Disease brain sections with anti-p97 antibody revealed an identical distribution to that of the transferrin receptor specific antibodies. In fact, both microglial cells and endothelial cells were labelled. In controls using secondary antibodies alone, however, no labelling of any cells or structures were seen in healthy or Alzheimer's Disease brain.

Electron microscopy was used to define the structures expressing p97 in capillaries. Brain tissue was prepared for electron microscopy as described in Example 10D. The DAB reaction products in sections stained with anti-p97 antibodies were found in the cytoplasm and attached to the membrane of endothelial cells (Figure 18). These results showing the presence of p97 on the surface of capillary cells and within the cytoplasm of capillary cells indicate a role for p97 in transport through brain endothelium. In Figure 18 capillary cells are labelled CP, red blood cells are labelled RBC and cytoplasm is labelled CYT.

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In summary, the expression of p97 and transferrin receptors by microglial cells of Alzheimer's Disease brain section appeared to be specific to this neurological disorder. No microglial labelling was observed in brain  
5 sections from PD, PSP, HD, MS, or ALS.

C. Expression of the p97 Antigen is Confined to Microglial Cells Associated with Amyloid Plaques

In order to examine the frequency and distribution of microglial cells which express the p97 or transferrin  
10 receptor molecules, double labelling experiments were undertaken with antibodies which react with  $\beta$ -amyloid protein ("BAP") or HLA-DR molecules. Figure 19I shows the double labelling of Beta Amyloid Protein (BAP) and HLA-DR. The BAP labelling appears as diffuse plaques. Microglial  
15 cells throughout the tissue are clearly labelled by the HLA-DR reactive antibody, including those cells not associated with amyloid plaques.

In double labelling studies with BAP reactive antibodies and p97 reactive antibodies a different  
20 labelling pattern appears. The p97 reactive antibody selectively identifies the subset of microglial cells associated with the senile plaque (Figure 19H). This appears to be consistent throughout the AD tissues studied to date. Furthermore, it appears that the microglial cells  
25 which express p97 are associated with blood vessels. The reason for this is unclear. Double labelling experiments using antibodies against BAP and the transferrin receptor reveals a similar pattern to that seen with the p97 reactive antibody.

30 In summary, microglial cells staining with anti-p97 or transferrin receptor were only detected in the cortices and hippocampus of AD cases or in PD plus AD cases. Compared with HLA-DR staining, which chiefly reacts with all microglia, anti-p97 antibody revealed smaller numbers  
35 of reactive microglia which were associated only with BAP. The processes of these microglia were frequently attached

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to capillaries.

D. Distribution of the p97 Molecule on/in Alzheimer's Brain Endothelial and Microglial Cells at the Electron Microscope Level

5 In order to establish the cellular structures which express the p97 molecule in AD brain, the distribution of the p97 molecule was determined by electron microscopy.

For electron microscopy, blocks of entorhinal cortex from two cases of AD were fixed in 1% glutaraldehyde/4%  
10 paraformaldehyde in 0.1M phosphate buffer, pH 7.4, for 24 hours at 4°C, followed by immersion in 15% sucrose in 0.1M phosphate buffer, pH 7.4, for several days at the same temperature. Sections were cut by vibratome at 50 mm thickness and incubated with anti-P97 (L235, 1:1,000) for  
15 5 days at 4°C. They were then treated with the appropriate Vectastain and ABC second antibody systems. After the diaminobenzidine (DAB) reaction, the sections were osmified, dehydrated and embedded in Epon. Ultra-thin sections were cut and examined with a Phillips EM201  
20 electron microscope, without counterstaining.

Electron-microscopically, DAB reactive products were seen in the membrane and cytoplasmic structures of endothelial cells labelled with anti p97 antibody (Figures 19D and 19E). In microglial cells, which are producing  
25 amyloid fibers, the reactivity of the antibody is limited to the cell membrane (Figures 19F and 19G). Thus it appears that the majority of the p97 molecule is expressed at the plasma membrane in both cell types. In addition, the p97 also appears to be expressed inside endothelial  
30 cells. This is consistent with the p97 molecule being made by microglial cells and being transported through endothelial cells.

E. PI-PLC Treatment of Alzheimer's Disease Brain Sections

35 As shown in Figures 19L (no PI-PLC treatment) and 19M

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(PI-PLC treatment), PI-PLC treatment prevents visualization of microglial cells in tissue sections stained with anti-p97 antibodies. As an additional control, anti-p97 antibodies were absorbed with p97, and  
5 used to stain Alzheimer's Disease brain sections. As shown in Figure 19N, no staining of microglial cells or blood vessels is evident.

#### EXAMPLE 11

##### 10 DETECTION OF P97 IN Alzheimer's DISEASE BRAIN (MEMBRANE AND CYTOPLASMIC FRACTIONS) BY WESTERN BLOTS

A Western blot analysis was carried out under non-reducing conditions in order to demonstrate the identity of the L235 antigen recognized in the tissue sections of Alzheimer's Disease brain and p97 containing cell lines  
15 (SK-MEL-28, p97aWTBce).

Briefly, SK-MEL-28, WTB and p97aWTBc3 cell cultures were grown up, washed and thereafter lysed in nonionic detergent 20 mM Tris-HCl pH 7.2, 150mM NaCl, 2mM EDTA, 1% NP-40, and 20 µg/ml PMSF. Membrane fractions and  
20 cytoplasmic fractions isolated from Alzheimer's Disease brains were homogenized and then precleared by centrifugation at 4°C at 10,000 x g for 10 minutes. The membrane and cytoplasmic fractions were then separated by a high-speed centrifugation at 4°C at 100,000 x g for 60  
25 minutes. The cell cytoplasmic and membrane samples were then analyzed by western blotting. Briefly, the proteins were separated on a 5-10% SDS-PAGE gel under non-reducing conditions, and then transferred onto Immobilon membranes (Millipore) by electroblotting. After incubation for 1  
30 hour in blocking buffer (2% BSA, 0.05% Tween-20,  $2.5 \times 10^{-4}$  M thimerosal in PBS), the membranes were washed 3 times in washing buffer (0.1% BSA, 0.05% Tween-20,  $2.5 \times 10^{-4}$  M thimerosal in PBS), and then incubated for 1 hour with L235 tissue culture supernatant as a first antibody.  
35 After further washing of the membranes, they were incubated for 1 hour with the secondary antibody, a

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1:10,000 dilution of donkey anti-mouse IgG conjugated with horseradish peroxidase (Jackson ImmunoResearch, 1:10,000 dilution). The specificity of the secondary antibody was determined after incubation with L235 as a first antibody  
5 or without a first antibody. After washing the proteins were detected by the chemiluminescence utilizing the ECL Western blotting detection system (Amersham).

Results are shown in Figure 20. A specific band with the same molecular weight as p97 in SK-MEL-28 and  
10 p97aWTBc3 cells can be detected in the membrane and cytoplasmic fraction from Alzheimer's Disease brain tissues. These results indicate that the same molecule is recognized by the L235 monoclonal antibody in brain tissue and on the cell lines, and the molecule is p97.

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**EXAMPLE 12****DETECTION OF P97 IN CEREBROSPINAL FLUID OF AD PATIENTS**

Samples of cerebrospinal fluid CSF (1-2ml) were obtained from eight AD patients and normal patients by spinal tap. Samples were concentrated by freeze drying at 135 °C. Samples were analysed using the BioRad minigel system as follows. Protein were separated on an SDS-PAGE gel (10% SDS running gel and 5% SDS stacking gel) under non-reducing conditions. Samples were boiled for 5 minutes at 95°C prior to loading in 20 µl of loading buffer (2M Tris-HCl, pH 8.8 containing sucrose and EDTA). Gels were removed and transferred to Immobilon membranes (Millipore) by electroblotting and dried overnight.

Western Blotting was carried out generally as described heretofore. More particularly, membranes were wet in methanol, incubated for 1 hour with western blocking buffer and incubated with 100 µl of L235 or OKT9 as a first antibody in 20 ml washing buffer for 1 hour at room temperature. Membranes were washed 3 times in washing buffer and incubated with secondary antibody: donkey anti-mouse IgG/HRPO (1:5000) or goat anti-mouse Ig/biotin (1:10,000) for 1 hour at room temperature. Membranes were washed 3 times in washing buffer and further in PBS, and incubated in streptavidin-horseradish peroxidase (1:5,000) for 30 minutes. After washing, the proteins were detected by chemiluminescence utilizing the ECL Western blotting detection system (Amersham). The results are shown in Figure 21. Bands corresponding to p-97 can be seen in Figure 21A showing the L235 filter. p-97 was not detected in the no first antibody control Figure 21C. Bands corresponding to p-97 were found exclusively in the L235 filter, showing the presence of p-97 in the CSF of Alzheimer's disease patients. Results from the control subjects showed no band corresponding to p-97. Figure 21B shows no band in the OKT9 filter. These results indicate that the presence of p-97 in the CSF may be a useful diagnostic for Alzheimer's disease patients.

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EXAMPLE 13

## IDENTIFICATION OF SOLUBLE FORMS OF P97/TRANSFERRIN

## RECEPTOR

SK-MEL-28 cells were pulsed with 200  $\mu$ Ci/ml of [ $^{35}$ S]  
5 methionine and washed twice with ice-cold biotinylation  
buffer. Cell surface antigens were biotinylated with NHS-  
LC-Biotin (100 $\mu$ g/ml) sulfosuccinimidyl 6-(biotin amido)  
hexanoate (Pierce). 2 ml of ice cold biotinylation  
10 solution was used for 5 minutes. The cells were washed  
three times in normal medium with an excess of cold  
methionine and chased for 0, 1, 4, 8, or 24 hours. After  
chase, the supernatant was collected and the cells were  
lysed in solubilization buffer with PMSF and Lys  
(0.1mg/ml). The cell supernatant and lysate were  
15 immunoprecipitated with L235 or OKT-9 as previously  
described and run under non-reducing conditions on a Bio-  
Rad mini-gel (10% SDS-PAGE). The protein was blotted on  
nitrocellulose membrane and the membrane was exposed to  
autoradiographic film for detection of radioactive  
20 proteins. Biotinylated proteins were detected by Western  
blots following the methodology previously described  
herein.

The results are shown in Figure 22. Figure 22A shows  
that p-97 labelled with [ $^{35}$ S]-methionine was detected in  
25 the supernatant (CS) and in the cell lysate (CL). Soluble  
p-97 was detected in the supernatant from 4-24 hours  
chase. Surprisingly, TR labelled with [ $^{35}$ S]-methionine was  
also detected in the supernatant (CS) and in the cell  
lysate (CL). The presence of labelled TR in the  
30 supernatant at 6-24 hours of chase suggests that there is  
a soluble form of TR. The results confirmed that the  
soluble form of p-97 does not originate from the membrane  
bound p-97, but must derive from another source. Figure  
22C shows that the soluble p-97 detected in the  
35 supernatant was not biotinylated and thus did not  
correspond to membrane-bound p-97 which had been released  
from the cell surface. Figure 22D shows the biotinylation



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labelling of TR. These results indicate the presence of soluble forms of both p-97 and the transferrin receptor.

#### EXAMPLE 14

##### SEMI-CONTINUOUS PROCESS TO PRODUCE P-97 FROM CHO CELLS

###### 5 A. Cell Line

The CHO cell line, WTB (obtained from Dr. Maxfield, New York University), was cotransfected with the p97 expression vector, pSV2p97a, and the G418 resistance vector, pWJ218 as described in Example 1. CHO cells were  
10 also adapted to suspension growth in serum free media, CHO-S-SFM (Gibco). Cells were cultured in either 75 cm<sup>2</sup> T-flasks (Gibco) or 250 and 500 mL spinner flasks (Bellco). All cell lines were incubated at 37C in a 5% CO<sub>2</sub> humidified atmosphere. When necessary, adherent lines  
15 were released by treatment with versene (saline solution of 2mg/mL EDTA). Cell density and viability were determined using a hemocytometer and trypan blue exclusion.

###### B. Monoclonal antibodies against p97

20 Two mouse hybridoma cell lines that produced monoclonal antibodies against p97 were grown in 500 mL spinner flasks. Hybridoma 33B6E4 (a gift from Dr. Shuen-Kuei Liao, Biotherapeutic, Franklin) was grown in DMEM supplemented with 10% FCS, 1% mercaptoethanol, 2 mM L-glutamine, and 0.8 mg/mL geneticin. L235 (ATCC HB8446 L235  
25 (M19)) was grown in RPMI supplemented with non-essential amino acids, 10% FCS, 1% mercaptoethanol, 2 mM L-glutamine, 2 mM L-proline, and 0.1 mg/mL penicillin/streptomycin. These hybridomas initially  
30 required a feeder layer of irradiated mouse embryonic fibroblast cells (ATCC X-56). L-235 cells were later selected to grow without the aid of a feeder layer and in the serum free media, HYBRIDOMA-SFM (Gibco).

Both cell lines were grown until their viability fell  
35 to below 50%. The cells were removed by centrifugation

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and the supernatant filtered through a 0.2  $\mu$ m membrane (VacuCap, Gelman Sciences, MI). The monoclonal antibodies were then purified using a protein G affinity column (MabTrap G, Pharmacia LKB Biotechnology Inc, N.J.), and later concentrated to 1-2 mg/mL using 10,000 MW ultrafilter (Centricon-10, Amicon Division, MA).

### C. Flow Cytometry

Cell surface expression of p97 was monitored using immunofluorescence labelling and a non-sorting flow cytometer analyzer (FACScan, Becton Dickinson) as described in Example 1. Cells were labelled with primary antibody (33B6E4) at 4C for 45 min. Cells were washed again in FACS buffer and incubated with the fluoresceinated secondary antibody (Goat anti-mouse IgG-FITC conjugate, Gibco) at 4C for 45 min. Cells were then washed in PBS and fixed in 1.5% (v/v) p-formaldehyde in PBS.

The FACScan flow cytometer measured 5000 events per sample. Data from individual experiments were compared to unstained controls and values expressed either as mean fluorescence per cell or in the case of PI-PLC treated cells, as percentages of untreated controls. By comparing the mean fluorescence/cell to p97 released by PI-PLC a linear relationship was found between mean fluorescence/cell and mean p97/cell.

### D. Measurement of p97 recovered in the PI-PLC solution

The concentration of p97 released by PI-PLC was determined using a Pandex fluorescent concentration analyzer (Idexx Ltd, Portland, OR). This is a rapid immunofluorescence technique, described in Jolley, M. J. Immunol. Methods 67:21-35, 1984, using carboxypolystyrene capture particles (0.8  $\mu$ m, 0.25% v/v, Idexx) coated with anti-p97 IgG (ATCC-HB8446 L235 (M-19)). This "activated" solid phase acts as a specific adsorbent for p97. A second anti-p97 IgG (33B6E4) was labelled with fluorescein

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isothiocyanate (Sigma Chemical Co.). Samples were assayed according to Jervis and Kilburn (Biotechnol. Prog. 7:28-32, 1991).

E. p97 Standard

5 p97 was purified from the supernatant of PI-PLC treated CHO cells by immunoaffinity chromatography. About 109 cells were treated with 1.0 mL of 0.1 U PI-PLC/mL in PBS and incubated for one hour at 37C. The cells were removed by centrifugation and the supernatant filtered  
10 through a 0.2  $\mu$ m membrane (Acrodisc 25, Gelman Sciences, MI). The filtrate was applied to a column (1X10 cm) of 33B6E4 Mab immobilized on Affi-Gel 10 (Bio-Rad, CA) that had been previously washed and regenerated in PBS, pH 7.2. The bound p97 was eluted with 0.1 M citric acid, pH  
15 3.0, followed by neutralization with 1 M Tris-HCl, pH 9.0. The purified p97 was further concentrated using a 30,000 MW ultrafiltration membrane (Centricon-30, Amicon Division, MA) then dialysed in PBS and sterile filtered. The p97 sample was shown to be pure according to SDS-  
20 PAGE. PhastGel 12.5% homogeneous polyacamide gel was run on the PhastSystem<sup>TM</sup> (Pharmacia LKB Biotechnology) and silver stained. The concentration of p97 was determined using the p97 extinction coefficient at 280 nm of  $\epsilon_{1\%} = 12.0 \text{ cm}^{-1}$ .

25 F. Production of PI-PLC

B. subtilis (BG2320) transfected with the gene for PI-PLC from B. thuringiensis was cultured using a procedure adapted from that described by Low, M.G. et al, J. Immunol. Methods 113:101-111, 1988) previously used to  
30 grow B. thuringiensis. The growth medium containing 10 g/L Polypeptone, 10 g/L yeast extract, 5 g/L NaCl, 0.4 g/L Na<sub>2</sub>HPO<sub>4</sub> and 15  $\mu$ g/mL chloramphenicol (pH adjusted to 7.0 with NaOH) was inoculated with 1.5-3% (v/v) of overnight preculture (initial O.D.<sub>600</sub> = 0.1). Cells were cultured  
35 in Erlenmeyer flasks and shaken at 150 rpm, 37C for 6 to

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12 h. Cells were removed by centrifugation and the supernatant filtered through a 0.2  $\mu$ m membrane (VacuCap, Gelman Sciences, MI). The supernatant was concentrated 20-fold using an ultrafiltration cell (Model 8400, Amicon Corp., MA) and a 10,000 MW YM10 ultrafilter (Amicon, MA). The concentrated enzyme solution was then washed two times with 5 volumes of PBS in the ultrafiltration cell. The enzyme solution was assayed and stored in 1 mL aliquots at -20C. When the enzyme was required, the frozen PI-PLC was rapidly thawed and diluted in PBS to the specified concentrations. All enzyme samples used in this study came from the same 2L batch fermentation.

#### G. PI-PLC Assay

One unit of PI-PLC is defined as the enzyme activity that hydrolyses 1  $\mu$ mol phosphatidylinositol per min at pH 7.5 and 37C. Phosphatidylinositol dissolved in detergent containing buffer was incubated with the PI-PLC sample. The diglyceride released was subsequently hydrolysed to free fatty acids and glycerol by the addition of lipase. The glycerol concentration was then determined enzymatically (Assay no. 5646, Boehringer Mannheim Biochemica).

#### H. PI-PLC treatment and Protein harvesting

Approximately 25 mL of 1-2x10<sup>6</sup> suspension cells/mL were centrifuged at 1300 rpm for 5 min and washed two times with 5 mL of PBS. The cells were then resuspended in 0.5 mL of PI-PLC in PBS (10 mU/mL) and incubated at 37C for 30 min with periodic agitation. Cells were again centrifuged and the supernatant recovered. The supernatant was further centrifuged at 10,000 rpm for 20 min, filtered through a 0.2  $\mu$ m membrane (Acrodisc 25, Gelman Sciences, MI) and stored at -20C prior to assaying for p97. A sample was concentrated 5-fold using a 3,000 MW ultrafiltration membrane (Centricon-3) for SDS-PAGE analysis.

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The enzyme treated cells were then washed two times with 15 mL PBS and once with 15 mL of CHO-S-SFM media. A sample of the cells were prepared for FACScan analysis and the rest resuspended in fresh CHO-S-SFM media at approximately  $0.5-1.0 \times 10^6$  cells/mL. The cells were then cultivated for a specified period before the protein harvest was repeated. Cell density, viability and glucose concentration of the media were determined prior to each protein harvest.

10 I. Expression of P97

CHO cells transfected with p97 cDNA and selected in geneticin were analyzed for p97 expression using flow cytometry. Approximately 2% of the bulk population expressed p97 (Figure 23). In Figure 23 graph A shows log scale fluorescence profiles of untransfected cell line WTB, B shows bulks transfected cells, C shows sorted cells and D shows subcloned cells. These cells expressing p97 were enriched ten-fold using fluorescence activated cell sorting. The sorted population (about 20% expressing cells) was further subcloned using limiting dilution and the highest p97 expressing clones isolated (Figure 23).

Suspension CHO clones expressing p97 were grown in 75 cm<sup>2</sup> T-flasks and 500 mL spinner flasks using serum-free medium. The growth profile for cells cultured in a 500 mL spinner flask is shown in Figure 24. The cells reached a peak concentration of over  $6 \times 10^6$  viable cells/mL in 6 days from an inoculum level of  $1.5 \times 10^5$  cells/mL with a mean doubling time of 26 hours. Viability fell sharply after 9 days when glucose levels had fallen to about 0.2 mg/mL. The cell surface expression of p97 per cell was monitored by flow cytometry analysis using FITC conjugated antibodies against p97 (Figure 25). Cell surface expression of p97 varied with the phase of cell growth. Maximal cell surface expression occurred after 3 days of growth at a cell density of  $1 \times 10^6$  cells/mL, after which there was a steady decline in cell specific

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expression. This decline could be partially explained by the reduction in average cell surface area as the culture viability decreases or cell death may release p97. The average cell diameter fell from 15  $\mu$ m during exponential growth to 13  $\mu$ m during the stationary phase with a corresponding reduction in viability from 99% to 90%.

A small amount of soluble, secreted p97 was detected in the supernatant at the end of the exponential phase of cell growth. When using the controlled release method of protein harvesting, secreted p97 would be discarded with the spent medium, and thus not recovered. However, this loss of p97 could be minimized if cells were harvested before the stationary phase of cell growth. This soluble p97 may result from the release of membrane bound protein, which could account for the reduction in average fluorescence per cell, or from the release of a soluble form of p97 that was not previously glipiated.

#### J. PI-PLC Treatment

Cell surface p97 was monitored by flow cytometry and the solubilized protein released by PI-PLC treatment was assayed by an immunoabsorption assay. The effect of the enzyme concentration on the percentage of protein removal for a 30 min incubation period is shown in Figure 26. Cells ( $10^8$  cells/mL) were treated with varying concentrations of enzyme in PBS. An enzyme concentration of 10 mU/ml was found to be sufficient for the removal of at least 90% of glipiated p97 from the cell surface. This is equivalent to a recovery of approximately 4 to  $5 \times 10^7$   $\mu$ g of p97 per viable cell. It was also observed that cells remained viable after the enzyme treatment. Cell viability remained above 95% for incubation times of up to one hour, after which viability decreased. The PI-PLC concentration of 10 mU/mL is equivalent to approximately 0.02  $\mu$ g/mL and, therefore, contributes a very low level of contaminating protein to p97 that has been harvested at over 1-40  $\mu$ g/mL concentrations(see below).

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The purity of the recovered p97 was estimated to be 30% based on Bio-Rad determination of total protein concentration using albumin as a standard. The contaminating proteins include other glypiated proteins removed from the cell surface by the action of PI-PLC.

#### K. Protein re-expression

In order to develop a semi-continuous method of harvesting p97, the recovery of PI-PLC treated cells and re-expression of p97 was investigated. Enzyme treated cells were washed two times in PBS and resuspended in fresh medium. After treatment with PI-PLC, suspension as well as adherent clones had doubling times identical to untreated cells. Surface re-expression of p97 was monitored using flow cytometry and the cells recovered 95% of their protein expression within 2 days (Figure 27).

#### L. Cyclic harvesting of p97

Having established that a 30 min incubation period with 10 mU/mL of PI-PLC in PBS was sufficient to remove at least 90% of the glypiated p97 from transfected CHO cells (Figure 26) and that cells retained their viability and were able to recover their p97 expression after enzyme treatment, it was next determined if the level of protein expression and cell viability would be adversely affected by repeated harvesting.

Cells were grown in suspension up to  $1-2 \times 10^6$  cells/mL and then centrifuged and washed with PBS. Approximately  $0.5 \times 10^8$  cells were treated with 1 mL of PI-PLC in PBS (10 mU/mL). After 30 min incubation, cells were again centrifuged and washed twice in PBS before resuspension in fresh medium at a concentration of  $1 \times 10^6$  cells/mL. The cells were subjected to further enzyme treatment at 24, 48 or 72 h intervals. Figure 28 shows the cumulative protein production per cell for all cycle times. The 24 h cycle produced the maximum amount of protein. However, based on medium utilization the 48 h cycle showed a

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greater yield of p97 (0.33 mg p97/g glucose consumed). Cell viability, cell density, cell specific p97 production and cell specific glucose consumption were monitored for each harvest cycle and were shown to be  
5 relatively stable for the duration of the experiments (Figure 29).

The concentration of p97 recovered after each 48 h harvest cycle is shown in Figure 30. Over a 44 day period with over 20 enzyme treatments the production of p97  
10 remained around 30 µg/ml. There appeared to be no drop in productivity over the duration of the experiment. It is also shown in Figure 30 that the suspension CHO cells secreted a steady level of 1 to 2 µg/ml of p97 into the growth medium. This may be considered a loss from a  
15 production point of view since the secreted p97 is discarded with the spent medium. However, it was observed that the level of basal secretion depends on the transfected CHO cell clone selected. For example another clone did not secrete detectable levels of p97 and could  
20 be used for production purposes.

The effectiveness of the various methods for the recovery of p97 are compared in Table 3. Direct addition of PI-PLC to adherent CHO cells growing with 10% serum resulted in the recovery of 1.2 µg/mL of p97 in medium  
25 containing a contaminating protein level of approximately 5.4 mg/mL. This represents a purity of 0.02% based on the total protein. When suspension cells growing in serum-free medium were treated with enzyme the results were substantially improved. Approximately 3 µg/ml of p97 was  
30 recovered in a media containing 380 µg/mL of protein, representing a purity of about 1%. The advantage of the cyclic harvest method was demonstrated by the finding that p97 was not only recovered at higher concentrations (30 µg/mL) but with a thousand-fold increase in purity to  
35 30%. The silver stained SDS-PAGE gel shown in Figure 31 compares p97 harvested into PI-PLC/PBS solution (Lanes 3 and 4) with p97 released directly into CHO-S-SFM serum



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free growth medium (Lane 2).

#### EXAMPLE 15

##### CELL SURFACE P97 BINDS IRON

The following cell lines were cultured as described herein: TRVb (no TR), TRVb-1 (human TR transfected), p97aTRVbc3 (human p-97 transfected) and p97aTRVb15 (human p-97 and TR transfected). The cells were washed and counted. Approximately  $6-10 \times 10^6$  cells were incubated with  $1 \mu\text{l}$   $^{55}\text{Fe}$  in  $\text{FeCl}$  salt for 0, 2, 6, 10 or 14 hours. 200  $\mu\text{l}$  of cells and medium were removed at each time point and centrifuged at maximum speed for 2 minutes at  $4^\circ\text{C}$  and the pelleted cells and supernatant separated into scintillation vials.  $^{55}\text{Fe}$  levels in vials for all time points were measured in a scintillation counter.

The experiments were repeated with the additional step of PI-PLC treatment (for 1 hour at  $37^\circ\text{C}$ ) of the cells and medium removed at each time point noted above. Following PI-PLC treatment the cells and medium were separated by centrifugation prior to scintillation counting as described above. The results are shown in Figures 32 and 33. Figures 32 and 33A shows that TRVB cell line containing p-97 had the highest counts over 6 hours, indicating that labelled iron is bound to p-97. After PI-PLC treatment the counts associated with the p-97 containing cell line decreased (Figure 33B), confirming that the cell surface, GPI-anchored p-97 binds iron.

From the foregoing, it will be appreciated that, although specific embodiments of the invention have been described herein for purposes of illustration, various modifications may be made without deviating from the spirit and scope of the invention. Accordingly, the invention is not limited except as by the appended.

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TABLE 1 - FACS RESULTS

<u>SAMPLE</u>		<u>FLUORESCENCE - converted linear value</u>
5	SK.MEL.28 - NFA	0.00
	SK.MEL.28 - + anti-p97	127.92
	SK.MEL.28 - + PI-PLC + anti-p97	4.52
	uninfected Sf9 - NFA	0.00
	uninfected Sf9 - + anti-p97	0.70
	uninfected Sf9 - + PI-PLC + anti-p97	0.96
10	AcMNPC (WT) - NFA	0.00
	AcMNPC (WT) - + anti-p97	-0.06
	AcMNPC (WT) - + PI-PLC + anti-p97	-0.06
15	p97 B-1-1 - NFA	0.00
	p97 B-1-1 - + anti-p97	111.66
	p97 B-1-1 - + PI-PLC + anti-p97	5.74
	p97 B-2-1 - NFA	0.00
	p97 B-2-1 - + anti-p97	97.38
	p97 B-2-1 - + PI-PLC + anti-p97	6.85

TABLE 2

20						
	<u>Antigen</u>	<u>Cells</u>	<u>Treatment</u>	<u>Fluorescence Intensity (% of Control)</u>		
25	p97	SK-MEL 28	PI-PLC	10.8	±	2.6
	P97	SK-MEL 28	Pronase	82.6	±	17.7
	TR	SK-MEL 28	PI-PLC	120.5	±	16.2
	TR	SK-MEL 28	Pronase	15.9	±	10.8
	Thy-1	EL-4	PI-PLC	32.5	±	6.4
	Thy-1	EL-4	Pronase	136.4	±	20.2
30	TR	EL-4	PI-PLC	93.1	±	9.4
	TR	EL-4	Pronase	1.9	±	1.0
	p97	p97aWTBc3	PI-PLC	6.8	±	4.0
	p97	p97aWTBc7	PI-PLC	7.5	±	5.1

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TABLE 3

5	Method	Cell Density	p97	Total Protein	P97 as % of Total Protein
		(10 <sup>6</sup> /mL)	(µg/mL)	(µg/mL)	
	Release into serum media - adherent	2.0	1.2	5400	0.02
10	Release into serum-free medium- suspension	6.0	3.0	380	0.8
15	Cyclic harvest of suspension cells	100	30	100	30

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## SEQUENCE LISTING

## (1) GENERAL INFORMATION:

- (i) APPLICANT: Jefferies, Wilfred A.  
McGeer, Patrick L.  
Rothenberger, Sylvia  
Food, Michael R.  
Yamada, Tatsuo
- (ii) TITLE OF INVENTION: Use of p97 and Iron Binding Proteins  
As Diagnostic and Therapeutic Agents
- (iii) NUMBER OF SEQUENCES: 11
- (iv) CORRESPONDENCE ADDRESS:
  - (A) ADDRESSEE: Bereskin & Parr
  - (B) STREET: 40 King Street West
  - (C) CITY: Toronto
  - (D) STATE: Ontario
  - (E) COUNTRY: Canada
  - (F) ZIP: M5H 3Y2
- (v) COMPUTER READABLE FORM:
  - (A) MEDIUM TYPE: Floppy disk
  - (B) COMPUTER: IBM PC compatible
  - (C) OPERATING SYSTEM: PC-DOS/MS-DOS
  - (D) SOFTWARE: PatentIn Release #1.0, Version #1.25
- (vi) CURRENT APPLICATION DATA:
  - (A) APPLICATION NUMBER:
  - (B) FILING DATE:
  - (C) CLASSIFICATION:
- (vii) ATTORNEY/AGENT INFORMATION:
  - (A) NAME: Linda M. Kurdydyk
  - (B) REGISTRATION NUMBER: 34,971
  - (C) REFERENCE/DOCKET NUMBER: 7390-001
- (ix) TELECOMMUNICATION INFORMATION:
  - (A) TELEPHONE: 416-364-7311
  - (B) TELEFAX: 416-361-1398
  - (C) TELEX: 06-23115

## (2) INFORMATION FOR SEQ ID NO:1:

- (i) SEQUENCE CHARACTERISTICS:
  - (A) LENGTH: 2368 base pairs
  - (B) TYPE: nucleic acid
  - (C) STRANDEDNESS: single
  - (D) TOPOLOGY: linear

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(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 61..117

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 118..2274

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

GCGGACTTCC TCGGACCCGG ACCCAGCCCC AGCCCGGCCC CAGCCAGCCC CGACGGCGCC  
60

ATG CGG GGT CCG AGC GGG GCT CTG TGG CTG CTC CTG GCT CTG CGC ACC  
108

Met Arg Gly Pro Ser Gly Ala Leu Trp Leu Leu Leu Ala Leu Arg Thr  
1 5 10 15

GTG CTC GGA GGC ATG GAG GTG CGG TGG TGC GCC ACC TCG GAC CCA GAG  
156

Val Leu Gly Gly Met Glu Val Arg Trp Cys Ala Thr Ser Asp Pro Glu  
1 5 10

CAG CAC AAG TGC GGC AAC ATG AGC GAG GCC TTC CGG GAA GCG GGC ATC  
204

Gln His Lys Cys Gly Asn Met Ser Glu Ala Phe Arg Glu Ala Gly Ile  
15 20 25

CAG CCC TCC CTC CTC TGC GTC CGG GGC ACC TCC GCC GAC CAC TGC GTC  
252

Gln Pro Ser Leu Leu Cys Val Arg Gly Thr Ser Ala Asp His Cys Val  
30 35 40 45

CAG CTC ATC GCG GCC CAG GAG GCT GAC GCC ATC ACT CTG GAT GGA GGA  
300

Gln Leu Ile Ala Ala Gln Glu Ala Asp Ala Ile Thr Leu Asp Gly Gly  
50 55 60

GCC ATC TAT GAG GCG GGA AAG GAG CAC GGC CTG AAG CCG GTG GTG GGC  
348

Ala Ile Tyr Glu Ala Gly Lys Glu His Gly Leu Lys Pro Val Val Gly  
65 70 75

GAA GTG TAC GAT CAA GAG GTC GGT ACC TCC TAT TAC GCC GTG GCT GTG  
396

Glu Val Tyr Asp Gln Glu Val Gly Thr Ser Tyr Tyr Ala Val Ala Val  
80 85 90

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GTC AGG AGG AGC TCC CAT GTG ACC ATT GAC ACC CTG AAA GGC GTG AAG  
 444  
 Val Arg Arg Ser Ser His Val Thr Ile Asp Thr Leu Lys Gly Val Lys  
 95 100 105

TCC TGC CAC ACG GGC ATC AAT CGC ACA GTG GGC TGG AAC GTG CCC GTG  
 492  
 Ser Cys His Thr Gly Ile Asn Arg Thr Val Gly Trp Asn Val Pro Val  
 110 115 120 125

GGC TAC CTG GTG GAG AGC GGC CGC CTC TCG GTG ATG GGC TGC GAT GTA  
 540  
 Gly Tyr Leu Val Glu Ser Gly Arg Leu Ser Val Met Gly Cys Asp Val  
 130 135 140

CTC AAA GCT GTC AGC GAC TAT TTT GGG GGC AGC TGC GTC CCG GGG GCA  
 588  
 Leu Lys Ala Val Ser Asp Tyr Phe Gly Gly Ser Cys Val Pro Gly Ala  
 145 150 155

GGA GAG ACC AGT TAC TCT GAG TCC CTC TGT CGC CTC TGC AGG GGT GAC  
 636  
 Gly Glu Thr Ser Tyr Ser Glu Ser Leu Cys Arg Leu Cys Arg Gly Asp  
 160 165 170

AGC TCT GGG GAA GGG GTG TGT GAC AAG AGC CCC CTG GAG AGA TAC TAC  
 684  
 Ser Ser Gly Glu Gly Val Cys Asp Lys Ser Pro Leu Glu Arg Tyr Tyr  
 175 180 185

GAC TAC AGC GGG GCC TTC CGG TGC CTG GCG GAA GGG GCA GGG GAC GTG  
 732  
 Asp Tyr Ser Gly Ala Phe Arg Cys Leu Ala Glu Gly Ala Gly Asp Val  
 190 195 200 205

GCT TTT GTG AAG CAC AGC ACG GTA CTG GAG AAC ACG GAT GGG AAG ACG  
 780  
 Ala Phe Val Lys His Ser Thr Val Leu Glu Asn Thr Asp Gly Lys Thr  
 210 215 220

CTT CCC TCC TGG GGC CAG GCC CTG CTG TCA CAG GAC TTC GAG CTG CTG  
 828  
 Leu Pro Ser Trp Gly Gln Ala Leu Leu Ser Gln Asp Phe Glu Leu Leu  
 225 230 235

TGC CGG GAT GGT AGC CGG GCC GAT GTC ACC GAG TGG AGG CAG TGC CAT  
 876  
 Cys Arg Asp Gly Ser Arg Ala Asp Val Thr Glu Trp Arg Gln Cys His  
 240 245 250

CTG GCC CGG GTG CCT GCT CAC GCC GTG GTG GTC CGG GCC GAC ACA GAT  
 924  
 Leu Ala Arg Val Pro Ala His Ala Val Val Val Arg Ala Asp Thr Asp

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255	260	265
GGG GGC CTC ATC TTC CGG CTG CTC AAC GAA GGC CAG CGT CTG TTC AGC 972		
Gly Gly Leu Ile Phe Arg Leu Leu Asn Glu Gly Gln Arg Leu Phe Ser 270 275 280 285		
CAC GAG GGC AGC AGC TTC CAG ATG TTC AGC TCT GAG GCC TAT GGC CAG 1020		
His Glu Gly Ser Ser Phe Gln Met Phe Ser Ser Glu Ala Tyr Gly Gln 290 295 300		
AAG GAT CTA CTC TTC AAA GAC TCT ACC TCG GAG CTT GTG CCC ATC GCC 1068		
Lys Asp Leu Leu Phe Lys Asp Ser Thr Ser Glu Leu Val Pro Ile Ala 305 310 315		
ACA CAG ACC TAT GAG GCG TGG CTG GGC CAT GAG TAC CTG CAC GCC ATG 1116		
Thr Gln Thr Tyr Glu Ala Trp Leu Gly His Glu Tyr Leu His Ala Met 320 325 330		
AAG GGT CTG CTC TGT GAC CCC AAC CGG CTG CCC CCC TAC CTG CGC TGG 1164		
Lys Gly Leu Leu Cys Asp Pro Asn Arg Leu Pro Pro Tyr Leu Arg Trp 335 340 345		
TGT GTG CTC TCC ACT CCC GAG ATC CAG AAG TGT GGA GAC ATG GCC GTG 1212		
Cys Val Leu Ser Thr Pro Glu Ile Gln Lys Cys Gly Asp Met Ala Val 350 355 360 365		
GCC TTC CGC CGG CAG CGC CTC AAG CCA GAG ATC CAG TGC GTG TCA GCC 1260		
Ala Phe Arg Arg Gln Arg Leu Lys Pro Glu Ile Gln Cys Val Ser Ala 370 375 380		
AAG TCC CCC CAA CAC TGC ATG GAG CGG ATC CAG GCT GAG CAG GTC GAC 1308		
Lys Ser Pro Gln His Cys Met Glu Arg Ile Gln Ala Glu Gln Val Asp 385 390 395		
GCT GTG ACC CTA AGT GGC GAG GAC ATT TAC ACG GCG GGG AAG AAG TAC 1356		
Ala Val Thr Leu Ser Gly Glu Asp Ile Tyr Thr Ala Gly Lys Lys Tyr 400 405 410		
GGC CTG GTT CCC GCA GCC GGC GAG CAC TAT GCC CCG GAA GAC AGC AGC 1404		
Gly Leu Val Pro Ala Ala Gly Glu His Tyr Ala Pro Glu Asp Ser Ser 415 420 425		
AAC TCG TAC TAC GTG GTG GCC GTG GTG AGA CGG GAC AGC TCC CAC GCC 1452		

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Asn Ser Tyr Tyr Val Val Ala Val Val Arg Arg Asp Ser Ser His Ala  
 430 435 440 445  
 TTC ACC TTG GAT GAG CTT CGG GGC AAG CGC TCC TGC CAC GCC GGT TTC  
 1500  
 Phe Thr Leu Asp Glu Leu Arg Gly Lys Arg Ser Cys His Ala Gly Phe  
 450 455 460  
 GGC AGC CCT GCA GGC TGG GAT GTC CCC GTG GGT GCC CTT ATT CAG AGA  
 1548  
 Gly Ser Pro Ala Gly Trp Asp Val Pro Val Gly Ala Leu Ile Gln Arg  
 465 470 475  
 GGC TTC ATC CGG CCC AAG GAC TGT GAC GTC CTC ACA GCA GTG AGC GAG  
 1596  
 Gly Phe Ile Arg Pro Lys Asp Cys Asp Val Leu Thr Ala Val Ser Glu  
 480 485 490  
 TTC TTC AAT GCC AGC TGC GTG CCC GTG AAC AAC CCC AAG AAC TAC CCC  
 1644  
 Phe Phe Asn Ala Ser Cys Val Pro Val Asn Asn Pro Lys Asn Tyr Pro  
 495 500 505  
 TCC TCG CTG TGT GCA CTG TGC GTG GGG GAC GAG CAG GGC CGC AAC AAG  
 1692  
 Ser Ser Leu Cys Ala Leu Cys Val Gly Asp Glu Gln Gly Arg Asn Lys  
 510 515 520 525  
 TGT GTG GGC AAC AGC CAG GAG CGG TAT TAC GGC TAC CGC GGC GCC TTC  
 1740  
 Cys Val Gly Asn Ser Gln Glu Arg Tyr Tyr Gly Tyr Arg Gly Ala Phe  
 530 535 540  
 AGG TGC CTG GTG GAG AAT GCG GGT GAC GTT GCC TTC GTC AGG CAC ACA  
 1788  
 Arg Cys Leu Val Glu Asn Ala Gly Asp Val Ala Phe Val Arg His Thr  
 545 550 555  
 ACC GTC TTT GAC AAC ACA AAC GGC CAC AAT TCC GAG CCC TGG GCT GCT  
 1836  
 Thr Val Phe Asp Asn Thr Asn Gly His Asn Ser Glu Pro Trp Ala Ala  
 560 565 570  
 GAG CTC AGG TCA GAG GAC TAT GAA CTG CTG TGC CCC AAC GGG GCC CGA  
 1884  
 Glu Leu Arg Ser Glu Asp Tyr Glu Leu Leu Cys Pro Asn Gly Ala Arg  
 575 580 585  
 GCC GAG GTG TCC CAG TTT GCA GCC TGC AAC CTG GCA CAG ATA CCA CCC  
 1932  
 Ala Glu Val Ser Gln Phe Ala Ala Cys Asn Leu Ala Gln Ile Pro Pro  
 590 595 600 605  
 CAC GCC GTG ATG GTC CGG CCC GAC ACC AAC ATC TTC ACC GTG TAT GGA

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1980  
 His Ala Val Met Val Arg Pro Asp Thr Asn Ile Phe Thr Val Tyr Gly  
                                 610                                615                                620

CTG CTG GAC AAG GCC CAG GAC CTG TTT GGA GAC GAC CAC AAT AAG AAC  
 2028  
 Leu Leu Asp Lys Ala Gln Asp Leu Phe Gly Asp Asp His Asn Lys Asn  
                                 625                                630                                635

GGG TTC AAA ATG TTC GAC TCC TCC AAC TAT CAT GGC CAA GAC CTG CTT  
 2076  
 Gly Phe Lys Met Phe Asp Ser Ser Asn Tyr His Gly Gln Asp Leu Leu  
                                 640                                645                                650

TTC AAG GAT GCC ACC GTC CGG GCG GTG CCT GTC GGA GAG AAA ACC ACC  
 2124  
 Phe Lys Asp Ala Thr Val Arg Ala Val Pro Val Gly Glu Lys Thr Thr  
                                 655                                660                                665

TAC CGC GGC TGG CTG GGG CTG GAC TAC GTG GCG GCG CTG GAA GGG ATG  
 2172  
 Tyr Arg Gly Trp Leu Gly Leu Asp Tyr Val Ala Ala Leu Glu Gly Met  
                                 670                                675                                680                                685

TCG TCT CAG CAG TGC TCG GGC GCA GCG GCC CCG GCG CCC GGG GCG CCC  
 2220  
 Ser Ser Gln Gln Cys Ser Gly Ala Ala Ala Pro Ala Pro Gly Ala Pro  
                                 690                                695                                700

CTG CTC CCG CTG CTG CTG CCC GCC CTC GCC GCC CGC CTG CTC CCG CCC  
 2268  
 Leu Leu Pro Leu Leu Leu Pro Ala Leu Ala Ala Arg Leu Leu Pro Pro  
                                 705                                710                                715

GCC CTC TGAGCCCGGC CGCCCCGCCC CAGAGCTCCG ATGCCCGCCC GGGGAGTTTC  
 2324  
 Ala Leu

CGCGGCGGCC TCTCGCGCTG CGGAATCCAG AAGGAAGCTC GCGA  
 2368

## (2) INFORMATION FOR SEQ ID NO:2:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 19 amino acids
- (B) TYPE: amino acid
- (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

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Met Arg Gly Pro Ser Gly Ala Leu Trp Leu Leu Leu Ala Leu Arg Thr  
 1 5 10 15  
 Val Leu Gly

## (2) INFORMATION FOR SEQ ID NO:3:

## (i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 719 amino acids  
 (B) TYPE: amino acid  
 (D) TOPOLOGY: linear

## (ii) MOLECULE TYPE: protein

## (xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

Gly Met Glu Val Arg Trp Cys Ala Thr Ser Asp Pro Glu Gln His Lys  
 1 5 10 15  
 Cys Gly Asn Met Ser Glu Ala Phe Arg Glu Ala Gly Ile Gln Pro Ser  
 20 25 30  
 Leu Leu Cys Val Arg Gly Thr Ser Ala Asp His Cys Val Gln Leu Ile  
 35 40 45  
 Ala Ala Gln Glu Ala Asp Ala Ile Thr Leu Asp Gly Gly Ala Ile Tyr  
 50 55 60  
 Glu Ala Gly Lys Glu His Gly Leu Lys Pro Val Val Gly Glu Val Tyr  
 65 70 75 80  
 Asp Gln Glu Val Gly Thr Ser Tyr Tyr Ala Val Ala Val Val Arg Arg  
 85 90 95  
 Ser Ser His Val Thr Ile Asp Thr Leu Lys Gly Val Lys Ser Cys His  
 100 105 110  
 Thr Gly Ile Asn Arg Thr Val Gly Trp Asn Val Pro Val Gly Tyr Leu  
 115 120 125  
 Val Glu Ser Gly Arg Leu Ser Val Met Gly Cys Asp Val Leu Lys Ala  
 130 135 140  
 Val Ser Asp Tyr Phe Gly Gly Ser Cys Val Pro Gly Ala Gly Glu Thr  
 145 150 155 160  
 Ser Tyr Ser Glu Ser Leu Cys Arg Leu Cys Arg Gly Asp Ser Ser Gly  
 165 170 175  
 Glu Gly Val Cys Asp Lys Ser Pro Leu Glu Arg Tyr Tyr Asp Tyr Ser  
 180 185 190

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Gly Ala Phe Arg Cys Leu Ala Glu Gly Ala Gly Asp Val Ala Phe Val  
 195 200 205  
 Lys His Ser Thr Val Leu Glu Asn Thr Asp Gly Lys Thr Leu Pro Ser  
 210 215 220  
 Trp Gly Gln Ala Leu Leu Ser Gln Asp Phe Glu Leu Leu Cys Arg Asp  
 225 230 235 240  
 Gly Ser Arg Ala Asp Val Thr Glu Trp Arg Gln Cys His Leu Ala Arg  
 245 250 255  
 Val Pro Ala His Ala Val Val Val Arg Ala Asp Thr Asp Gly Gly Leu  
 260 265 270  
 Ile Phe Arg Leu Leu Asn Glu Gly Gln Arg Leu Phe Ser His Glu Gly  
 275 280 285  
 Ser Ser Phe Gln Met Phe Ser Ser Glu Ala Tyr Gly Gln Lys Asp Leu  
 290 295 300  
 Leu Phe Lys Asp Ser Thr Ser Glu Leu Val Pro Ile Ala Thr Gln Thr  
 305 310 315 320  
 Tyr Glu Ala Trp Leu Gly His Glu Tyr Leu His Ala Met Lys Gly Leu  
 325 330 335  
 Leu Cys Asp Pro Asn Arg Leu Pro Pro Tyr Leu Arg Trp Cys Val Leu  
 340 345 350  
 Ser Thr Pro Glu Ile Gln Lys Cys Gly Asp Met Ala Val Ala Phe Arg  
 355 360 365  
 Arg Gln Arg Leu Lys Pro Glu Ile Gln Cys Val Ser Ala Lys Ser Pro  
 370 375 380  
 Gln His Cys Met Glu Arg Ile Gln Ala Glu Gln Val Asp Ala Val Thr  
 385 390 395 400  
 Leu Ser Gly Gln Asp Ile Tyr Thr Ala Gly Lys Lys Tyr Gly Leu Val  
 405 410 415  
 Pro Ala Ala Gly Glu His Tyr Ala Pro Glu Asp Ser Ser Asn Ser Tyr  
 420 425 430  
 Tyr Val Val Ala Val Val Arg Arg Asp Ser Ser His Ala Phe Thr Leu  
 435 440 445  
 Asp Glu Leu Arg Gly Lys Arg Ser Cys His Ala Gly Phe Gly Ser Pro  
 450 455 460  
 Ala Gly Trp Asp Val Pro Val Gly Ala Leu Ile Gln Arg Gly Phe Ile  
 465 470 475 480  
 Arg Pro Lys Asp Cys Asp Val Leu Thr Ala Val Ser Glu Phe Phe Asn

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				485					490							495			
Ala	Ser	Cys	Val	Pro	Val	Asn	Asn	Pro	Lys	Asn	Tyr	Pro	Ser	Ser	Leu				
			500					505					510						
Cys	Ala	Leu	Cys	Val	Gly	Asp	Glu	Gln	Gly	Arg	Asn	Lys	Cys	Val	Gly				
		515					520					525							
Asn	Ser	Gln	Glu	Arg	Tyr	Tyr	Gly	Tyr	Arg	Gly	Ala	Phe	Arg	Cys	Leu				
	530					535					540								
Val	Glu	Asn	Ala	Gly	Asp	Val	Ala	Phe	Val	Arg	His	Thr	Thr	Val	Phe				
545					550					555					560				
Asp	Asn	Thr	Asn	Gly	His	Asn	Ser	Glu	Pro	Trp	Ala	Ala	Glu	Leu	Arg				
				565					570					575					
Ser	Glu	Asp	Tyr	Glu	Leu	Leu	Cys	Pro	Asn	Gly	Ala	Arg	Ala	Glu	Val				
			580					585					590						
Ser	Gln	Phe	Ala	Ala	Cys	Asn	Leu	Ala	Gln	Ile	Pro	Pro	His	Ala	Val				
		595					600					605							
Met	Val	Arg	Pro	Asp	Thr	Asn	Ile	Phe	Thr	Val	Tyr	Gly	Leu	Leu	Asp				
	610					615					620								
Lys	Ala	Gln	Asp	Leu	Phe	Gly	Asp	Asp	His	Asn	Lys	Asn	Gly	Phe	Lys				
625					630					635					640				
Met	Phe	Asp	Ser	Ser	Asn	Tyr	His	Gly	Gln	Asp	Leu	Leu	Phe	Lys	Asp				
				645					650					655					
Ala	Thr	Val	Arg	Ala	Val	Pro	Val	Gly	Glu	Lys	Thr	Thr	Tyr	Arg	Gly				
			660					665					670						
Trp	Leu	Gly	Leu	Asp	Tyr	Val	Ala	Ala	Leu	Glu	Gly	Met	Ser	Ser	Gln				
		675					680					685							
Gln	Cys	Ser	Gly	Ala	Ala	Ala	Pro	Ala	Pro	Gly	Ala	Pro	Leu	Leu	Pro				
	690					695					700								
Leu	Leu	Leu	Pro	Ala	Leu	Ala	Ala	Arg	Leu	Leu	Pro	Pro	Ala	Leu					
705					710						715								

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 14 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

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(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GCGGACTTCC TCGG  
14

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 13 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

TCGCGAGCTT CCT  
13

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 20 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

CTCAGAGGGC CGCTGCGCCC  
20

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 21 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

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(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

CCAGCGCAGC TAGCGGGGCA G  
21

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 27 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

ACACCAGCGC AGCTCGAGGG GCAGCCG  
27

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 37 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

CGCGTACGTA TGATCACCCG AGCACTGCTG AGACGAC  
37

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 40 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

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(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

GCGCTACGTA CTCGAGGCCC CAGCCAGCCC CGACGGCGCC  
40

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:  
(A) LENGTH: 41 base pairs  
(B) TYPE: nucleic acid  
(C) STRANDEDNESS: single  
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(iii) HYPOTHETICAL: NO

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

CGCGTACGTA TGATCATCAG CCCGAGCACT GCTGAGACGA C  
41

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We Claim:

1. Isolated p97 having a glycosyl-phosphatidylinositol anchor.
2. A method of preparing a cleaved form of the isolated p97 having a glycosyl-phosphatidylinositol anchor as claimed in claim 1, comprising incubating a cell which expresses the isolated p97 on its surface with an enzyme that cleaves glycosyl-phosphatidylinositol (GPI) anchors, to produce the cleaved form of the isolated p97, and isolating the cleaved form.
3. An isolated soluble p97 which is hydrophilic.
4. The isolated soluble p97 as claimed in claim 3 labelled with a label selected from the group consisting of fluorescent molecules, luminescent molecules, enzymes, substances having therapeutic activity, toxins, and radionuclides.
5. A method for treating conditions involving disturbances in iron metabolism comprising administering an iron modulating amount of p97 or a stimulant, agonist or antagonist of p97.
6. A method for treating conditions involving disturbances in iron metabolism comprising administering an iron modulating amount of p97, or a stimulant, agonist or antagonist thereof.
7. A method for identifying a stimulant, agonist or antagonist of p97 comprising (a) reacting a substance suspected of being a stimulant, agonist or antagonist of p97 with p97 and iron under conditions such that p97 is capable of binding the iron, measuring the amount of p97 bound to iron, and determining the effect of the substance



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by comparing the amount of p97 bound to iron with an amount determined for a control; (b) reacting a substance suspected of being a stimulant, agonist or antagonist of p97 with p97 and transferrin receptor under conditions such that p97 is capable of binding to the transferrin receptor, measuring the amount of p97 bound to transferrin receptor, and determining the effect of the substance by comparing the amount p97 bound to transferrin receptor with an amount determined for a control; or (c) reacting a substance suspected of being a stimulant, agonist or antagonist of p97 with a cell which expresses p97, measuring the amount of p97 expressed by the cell, and determining the effect of the substance by comparing the amount of expression of p97 with an amount determined for a control.

8. A composition for delivering an agent across the blood brain barrier comprising p97 conjugated to the agent, a substance which is capable of specifically binding to p97 conjugated to the agent, or a p97 fusion protein containing p97 or the N-terminal or C-terminal portion of p97 sequence fused to the agent, and a pharmaceutically acceptable carrier or diluent.

9. A method of delivering an agent across the blood brain barrier comprising peripherally administering a composition as claimed in claim 8.

10. A method for monitoring Alzheimer's Disease, comprising detecting the presence of soluble p97 in a sample from a patient.

11. A method for monitoring Alzheimer's Disease, as claimed in claim 10 which is a radioimmunoassay, competitive assay, or enzyme linked immunosorbant assay.

12. A method for monitoring Alzheimer's Disease,

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comprising detecting the presence of transferrin receptors, or detecting the presence of p97 on microglial cells associated with amyloid plaques in a patient.

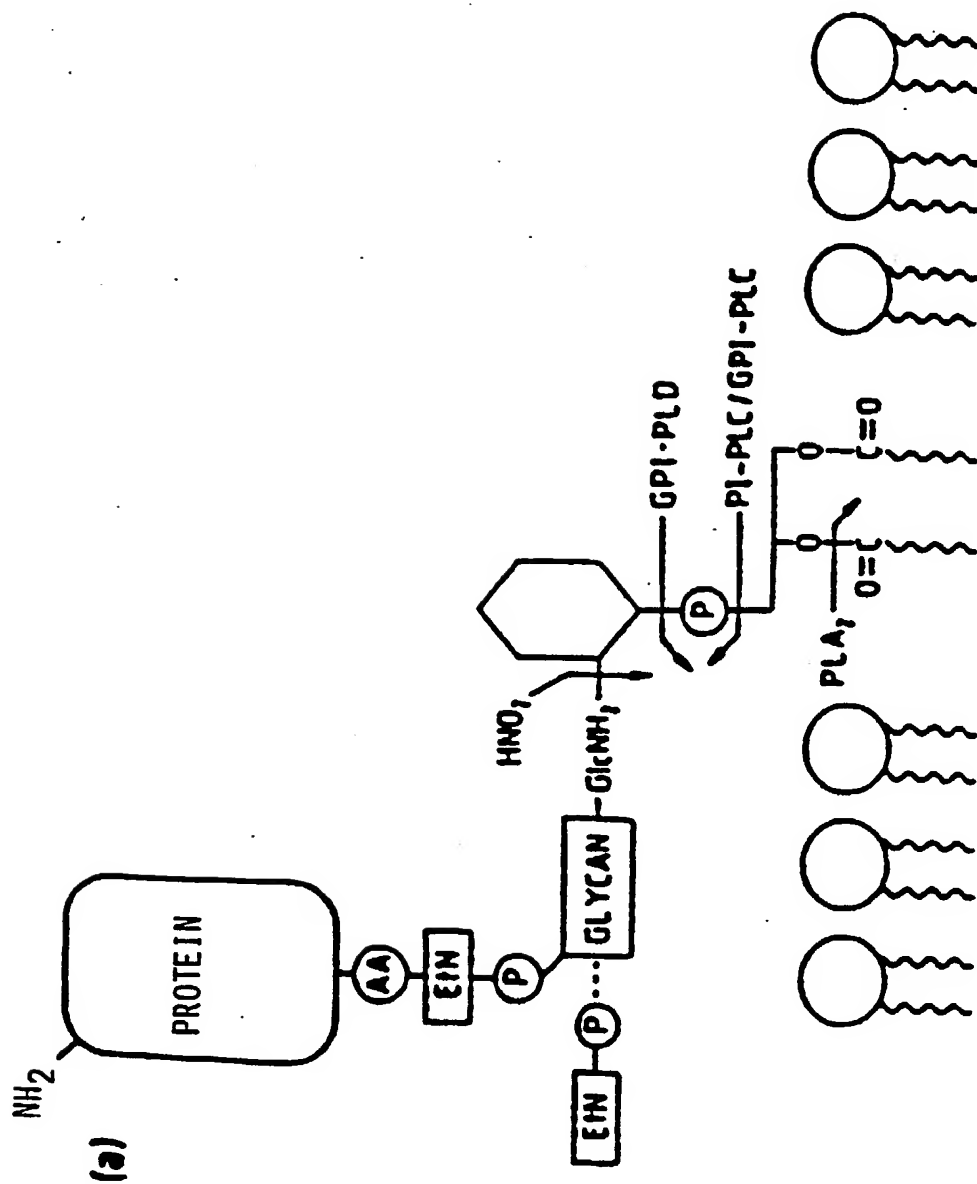
13. A method for treating Alzheimer's Disease in a patient comprising administering p97, transferrin, transferrin receptor, antibody specific to p97 or transferrin receptor, or an iron chelator to deplete iron in the brain of the patient.

14. A method for treating Alzheimer's Disease comprising the step of administering to a patient p97, transferrin receptor, or a substance which is capable of binding to p97 or labelled transferrin receptor which are labelled with a substance having therapeutic activity or a toxin.

15. A method of purifying microglial cells associated with Alzheimer's Disease beta amyloid plaques comprising reacting a sample suspected of containing microglial cells associated with Alzheimer's Disease beta amyloid plaques with a substance which specifically binds p97 or transferrin receptor under conditions such that the microglial cells bind to the substance; isolating the microglial cells bound to the substance; and optionally transforming the purified microglial cells to produce a cell line.

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FIGURE 1



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## FIGURE 2A

GCGGACTTCC TCGGACCCGG ACCCAGCCCC AGCCCGGCCC CAGCCAGCCC CGACGGCGCC	60
ATG CGG GGT CCG AGC GGG GCT CTG TGG CTG CTC CTG GCT CTG CGC ACC Met Arg Gly Pro Ser Gly Ala Leu Trp Leu Leu Leu Ala Leu Arg Thr	108
1 5 10 15	
GTG CTC GGA GGC ATG GAG GTG CGG TGG TGC GCC ACC TCG GAC CCA GAG Val Leu Gly Gly Met Glu Val Arg Trp Cys Ala Thr Ser Asp Pro Glu	156
1 5 10	
CAG CAC AAG TGC GGC AAC ATG AGC GAG GCC TTC CGG GAA GCG GGC ATC Gln His Lys Cys Gly Asn Met Ser Glu Ala Phe Arg Glu Ala Gly Ile	204
15 20 25	
CAG CCC TCC CTC CTC TGC GTC CGG GGC ACC TCC GCC GAC CAC TGC GTC Gln Pro Ser Leu Leu Cys Val Arg Gly Thr Ser Ala Asp His Cys Val	252
30 35 40 45	
CAG CTC ATC GCG GCC CAG GAG GCT GAC GCC ATC ACT CTG GAT GGA GGA Gln Leu Ile Ala Ala Gln Glu Ala Asp Ala Ile Thr Leu Asp Gly Gly	300
50 55 60	
GCC ATC TAT GAG GCG GGA AAG GAG CAC GGC CTG AAG CCG GTG GTG GGC Ala Ile Tyr Glu Ala Gly Lys Glu His Gly Leu Lys Pro Val Val Gly	348
65 70 75	
GAA GTG TAC GAT CAA GAG GTC GGT ACC TCC TAT TAC GCC GTG GCT GTG Glu Val Tyr Asp Gln Glu Val Gly Thr Ser Tyr Tyr Ala Val Ala Val	396
80 85 90	
GTC AGC AGG AGC TCC CAT GTG ACC ATT GAC ACC CTG AAA GGC GTG AAG Val Arg Arg Ser Ser His Val Thr Ile Asp Thr Leu Lys Gly Val Lys	444
95 100 105	

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FIGURE 2B

TCC TGC CAC ACG GGC ATC AAT CGC ACA GTG GGC TGG AAC GTG CCC GTG Ser Cys His Thr Gly Ile Asn Arg Thr Val Gly Trp Asn Val Pro Val 110 115 120 125	492
GGC TAC CTG GTG GAG AGC GGC CGC CTC TCG GTG ATG GGC TGC GAT GTA Gly Tyr Leu Val Glu Ser Gly Arg Leu Ser Val Met Gly Cys Asp Val 130 135 140	540
CTC AAA GCT GTC AGC GAC TAT TTT GGG GGC AGC TGC GTC CCG GGG GCA Leu Lys Ala Val Ser Asp Tyr Phe Gly Gly Ser Cys Val Pro Gly Ala 145 150 155	588
GGA GAG ACC AGT TAC TCT GAG TCC CTC TGT CGC CTC TGC AGG GGT GAC Gly Glu Thr Ser Tyr Ser Glu Ser Leu Cys Arg Leu Cys Arg Gly Asp 160 165 170	636
AGC TCT GGG GAA GGG GTG TGT GAC AAG AGC CCC CTG GAG AGA TAC TAC Ser Ser Gly Glu Gly Val Cys Asp Lys Ser Pro Leu Glu Arg Tyr Tyr 175 180 185	684
GAC TAC AGC GGG GCC TTC CGG TGC CTG GCG GAA GGG GCA GGG GAC GTG Asp Tyr Ser Gly Ala Phe Arg Cys Leu Ala Glu Gly Ala Gly Asp Val 190 195 200 205	732
GCT TTT GTG AAG CAC AGC ACG GTA CTG GAG AAC ACG GAT GGG AAG ACG Ala Phe Val Lys His Ser Thr Val Leu Glu Asn Thr Asp Gly Lys Thr 210 215 220	780
CTT CCC TCC TGG GGC CAG GCC CTG CTG TCA CAG GAC TTC GAG CTG CTG Leu Pro Ser Trp Gly Gln Ala Leu Leu Ser Gln Asp Phe Glu Leu Leu 225 230 235	828
TGC CGG GAT GGT AGC CGG GCC GAT GTC ACC GAG TGG AGG CAG TGC CAT Cys Arg Asp Gly Ser Arg Ala Asp Val Thr Glu Trp Arg Gln Cys His 240 245 250	876

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## FIGURE 2C

CTG GCC CGG GTG CCT GCT CAC GCC GTG GTG GTC CGG GCC GAC ACA GAT	924
Leu Ala Arg Val Pro Ala His Ala Val Val Val Arg Ala Asp Thr Asp	
255 260 265	
GGG GGC CTC ATC TTC CGG CTG CTC AAC GAA GGC CAG CGT CTG TTC AGC	972
Gly Gly Leu Ile Phe Arg Leu Leu Asn Glu Gly Gln Arg Leu Phe Ser	
270 275 280 285	
CAC GAG GGC AGC AGC TTC CAG ATG TTC AGC TCT GAG GCC TAT GGC CAG	1020
His Glu Gly Ser Ser Phe Gln Met Phe Ser Ser Glu Ala Tyr Gly Gln	
290 295 300	
AAG GAT CTA CTC TTC AAA GAC TCT ACC TCG GAG CTT GTG CCC ATC GCC	1068
Lys Asp Leu Leu Phe Lys Asp Ser Thr Ser Glu Leu Val Pro Ile Ala	
305 310 315	
ACA CAG ACC TAT GAG GCG TGG CTG GGC CAT GAG TAC CTG CAC GCC ATG	1116
Thr Gln Thr Tyr Glu Ala Trp Leu Gly His Glu Tyr Leu His Ala Met	
320 325 330	
AAG GGT CTG CTC TGT GAC CCC AAC CGG CTG CCC CCC TAC CTG CGC TGG	1164
Lys Gly Leu Leu Cys Asp Pro Asn Arg Leu Pro Pro Tyr Leu Arg Trp	
335 340 345	
TGT GTG CTC TCC ACT CCC GAG ATC CAG AAG TGT GGA GAC ATG GCC GTG	1212
Cys Val Leu Ser Thr Pro Glu Ile Gln Lys Cys Gly Asp Met Ala Val	
350 355 360 365	
GCC TTC CGC CGG CAG CGC CTC AAG CCA GAG ATC CAG TGC GTG TCA GCC	1260
Ala Phe Arg Arg Gln Arg Leu Lys Pro Glu Ile Gln Cys Val Ser Ala	
370 375 380	
AAG TCC CCC CAA CAC TGC ATG GAG CGG ATC CAG GCT GAG CAG GTC GAC	1308
Lys Ser Pro Gln His Cys Met Glu Arg Ile Gln Ala Glu Gln Val Asp	
385 390 395	

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## FIGURE 2D

GCT GTG ACC CTA AGT GGC GAG GAC ATT TAC ACG GCG GGG AAG AAG TAC Ala Val Thr Leu Ser Gly Glu Asp Ile Tyr Thr Ala Gly Lys Lys Tyr 400 405 410	1356
GGC CTG GTT CCC GCA GCC GGC GAG CAC TAT GCC CCG GAA GAC AGC AGC Gly Leu Val Pro Ala Ala Gly Glu His Tyr Ala Pro Glu Asp Ser Ser 415 420 425	1404
AAC TCG TAC TAC GTG GTG GCC GTG GTG AGA CGG GAC AGC TCC CAC GCC Asn Ser Tyr Tyr Val Val Ala Val Val Arg Arg Asp Ser Ser His Ala 430 435 440 445	1452
TTC ACC TTG GAT GAG CTT CGG GGC AAG CGC TCC TGC CAC GCC GGT TTC Phe Thr Leu Asp Glu Leu Arg Gly Lys Arg Ser Cys His Ala Gly Phe 450 455 460	1500
GGC AGC CCT GCA GGC TGG GAT GTC CCC GTG GGT GCC CTT ATT CAG AGA Gly Ser Pro Ala Gly Trp Asp Val Pro Val Gly Ala Leu Ile Gln Arg 465 470 475	1548
GGC TTC ATC CGG CCC AAG GAC TGT GAC GTC CTC ACA GCA GTG AGC GAG Gly Phe Ile Arg Pro Lys Asp Cys Asp Val Leu Thr Ala Val Ser Glu 480 485 490	1596
TTC TTC AAT GCC AGC TGC GTG CCC GTG AAC AAC CCC AAG AAC TAC CCC Phe Phe Asn Ala Ser Cys Val Pro Val Asn Asn Pro Lys Asn Tyr Pro 495 500 505	1644
TCC TCG CTG TGT GCA CTG TGC GTG GGG GAC GAG CAG GGC CGC AAC AAG Ser Ser Leu Cys Ala Leu Cys Val Gly Asp Glu Gln Gly Arg Asn Lys 510 515 520 525	1692
TGT GTG GGC AAC AGC CAG GAG CGG TAT TAC GGC TAC CGC GGC GCC TTC Cys Val Gly Asn Ser Gln Glu Arg Tyr Tyr Gly Tyr Arg Gly Ala Phe 530 535 540	1740

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## FIGURE 2E

AGG TGC CTG GTG GAG AAT GCG GGT GAC GTT GCC TTC GTC AGG CAC ACA Arg Cys Leu Val Glu Asn Ala Gly Asp Val Ala Phe Val Arg His Thr 545 550 555	1788
ACC GTC TTT GAC AAC ACA AAC GGC CAC AAT TCC GAG CCC TGG GCT GCT Thr Val Phe Asp Asn Thr Asn Gly His Asn Ser Glu Pro Trp Ala Ala 560 565 570	1836
GAG CTC AGG TCA GAG GAC TAT GAA CTG CTG TGC CCC AAC GGG GCC CGA Glu Leu Arg Ser Glu Asp Tyr Glu Leu Leu Cys Pro Asn Gly Ala Arg 575 580 585	1884
GCC GAG GTG TCC CAG TTT GCA GCC TGC AAC CTG GCA CAG ATA CCA CCC Ala Glu Val Ser Gln Phe Ala Ala Cys Asn Leu Ala Gln Ile Pro Pro 590 595 600 605	1932
CAC GCC GTG ATG GTC CGG CCC GAC ACC AAC ATC TTC ACC GTG TAT GGA His Ala Val Met Val Arg Pro Asp Thr Asn Ile Phe Thr Val Tyr Gly 610 615 620	1980
CTG CTG GAC AAG GCC CAG GAC CTG TTT GGA GAC GAC CAC AAT AAG AAC Leu Leu Asp Lys Ala Gln Asp Leu Phe Gly Asp Asp His Asn Lys Asn 625 630 635	2028
GGG TTC AAA ATG TTC GAC TCC TCC AAC TAT CAT GGC CAA GAC CTG CTT Gly Phe Lys Met Phe Asp Ser Asn Tyr His Gly Gln Asp Leu Leu 640 645 650	2076
TTC AAG GAT GCC ACC GTC CGG GCG GTG CCT GTC GGA GAG AAA ACC ACC Phe Lys Asp Ala Thr Val Arg Ala Val Pro Val Gly Glu Lys Thr Thr 655 660 665	2124
TAC CGC GGC TGG CTG GGG CTG GAC TAC GTG GCG GCG CTG GAA GGG ATG Tyr Arg Gly Trp Leu Gly Leu Asp Tyr Val Ala Ala Leu Glu Gly Met 670 675 680 685	2172

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## FIGURE 2F

TCG TCT CAG CAG TGC TCG GGC GCA GCG GCC CCG GCG CCC GGG GCG CCC	2220
Ser Ser Gln Gln Cys Ser Gly Ala Ala Ala Pro Ala Pro Gly Ala Pro	
690 695 700	
CTG CTC CCG CTG CTG CTG CCC GCC CTC GCC GCC GCG CTG CTC CCG CCC	2268
Leu Leu Pro Leu Leu Leu Pro Ala Leu Ala Ala Arg Leu Leu Pro Pro	
705 710 715	
GCC CTC TGAGCCCGGC CGCCCGGCC CAGAGCTCCG ATGCCCGCCC GGGGAGTTTC	2324
Ala Leu	
CGCGGCGGCC TCTCGCGCTG CGGAATCCAG AAGGAAGCTC GCGA	2368

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FIGURE 3

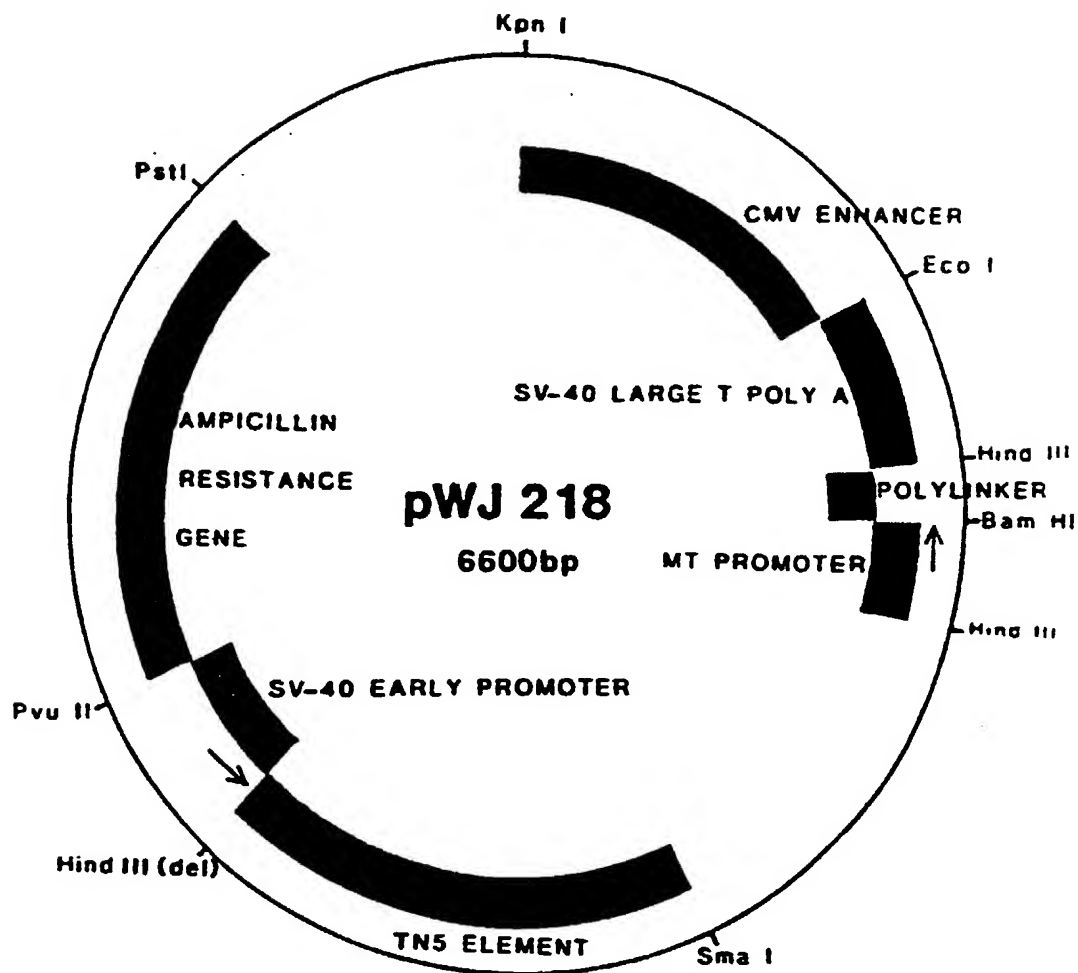


FIGURE 4

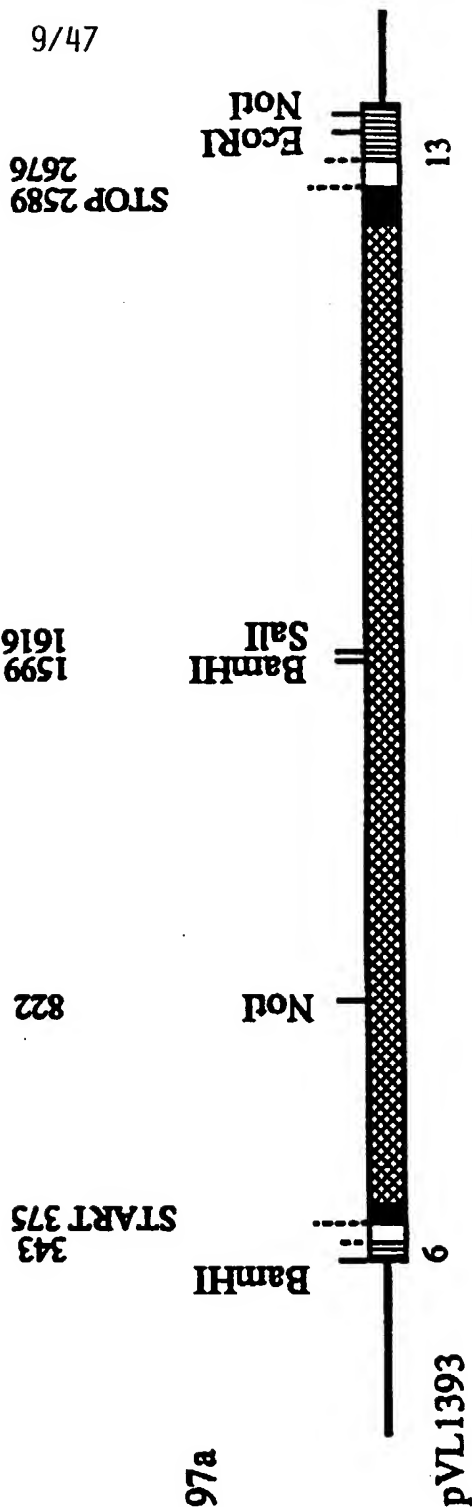
MAP:



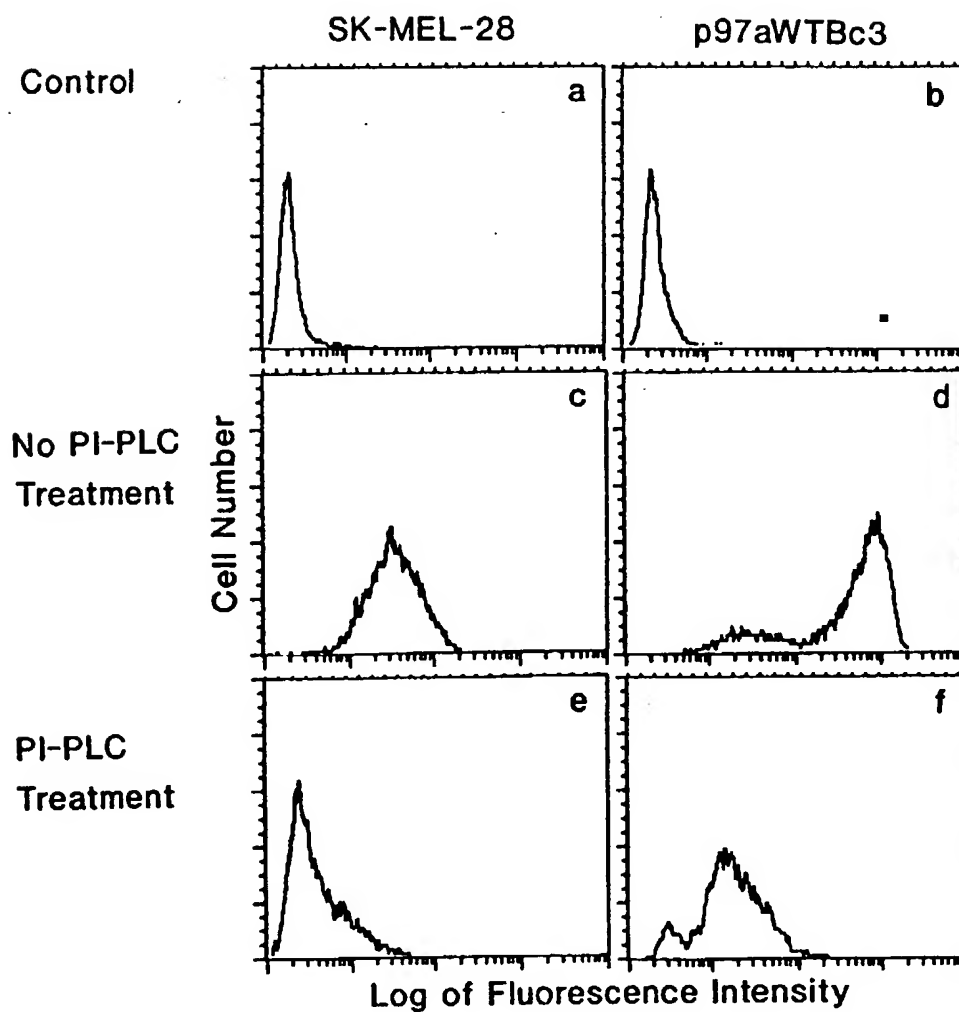
coding region human p97 cDNA (#375-2589)  
 Leader sequence/hydrophobic domain of p97  
 5' and 3' UTR region of p97 cDNA (not complete)  
 Multiple cloning sites of pVL 1393

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# pSV2p97a

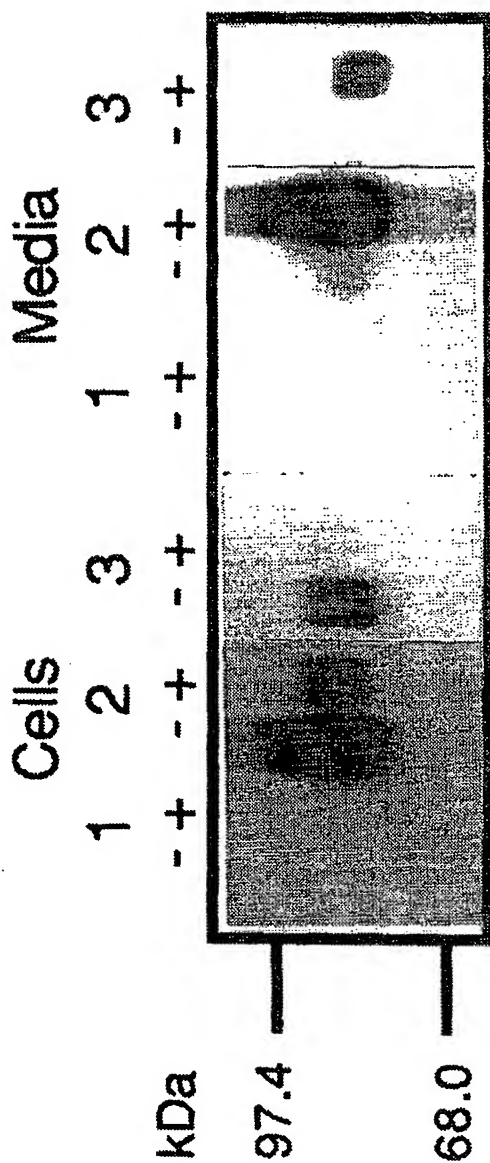


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**FIGURE 5****SUBSTITUTE SHEET**

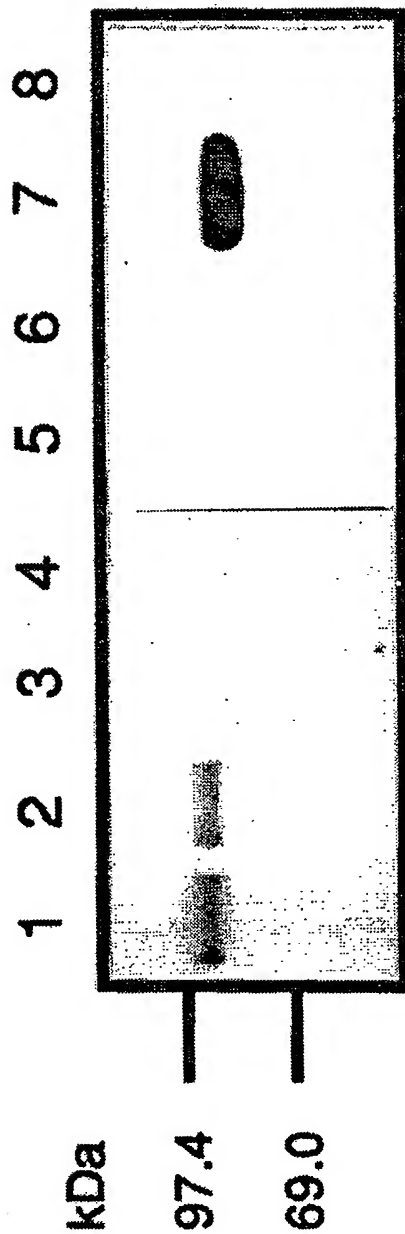
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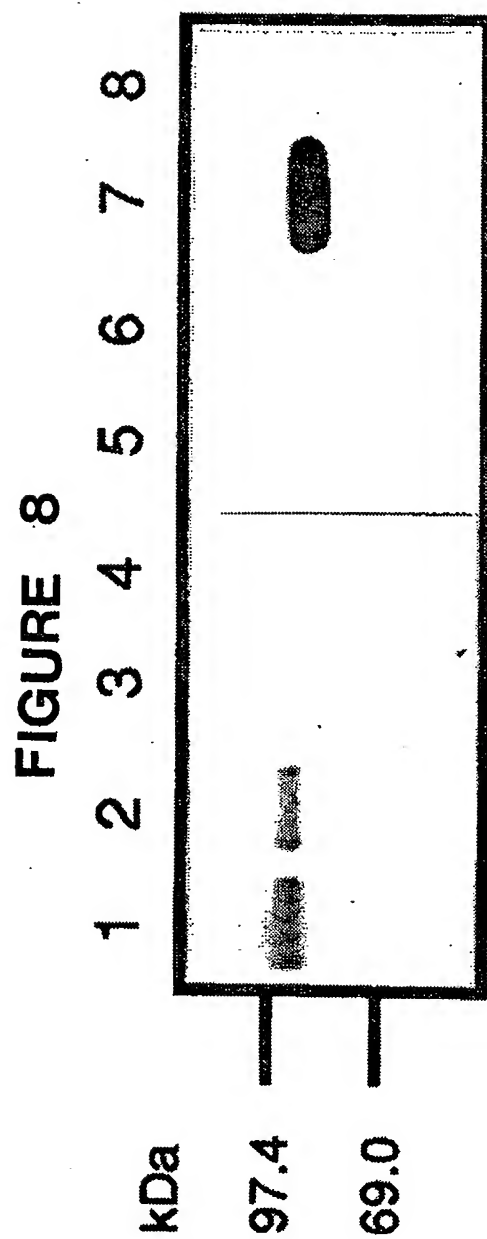
FIGURE 6



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FIGURE 7



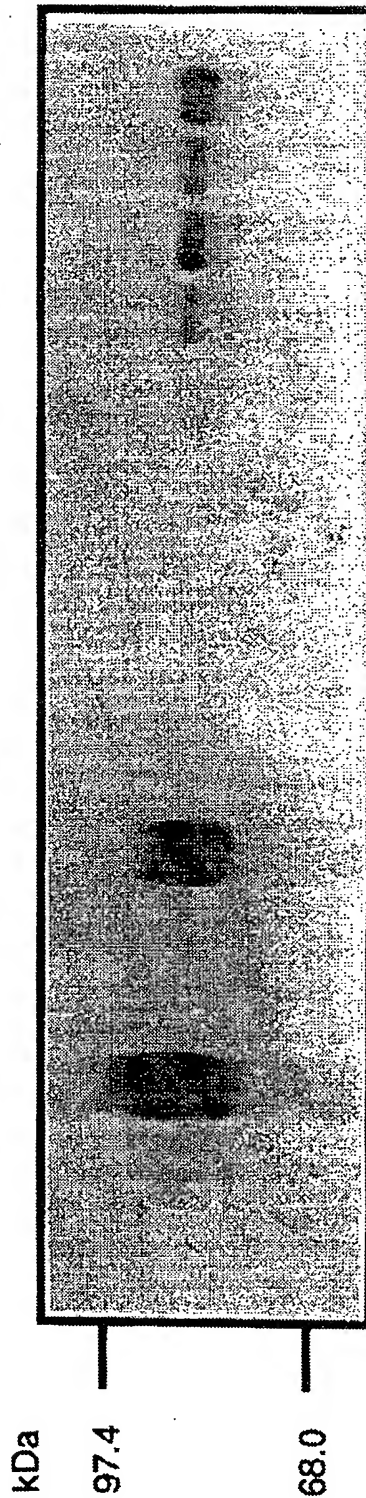


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FIGURE 9

p97 TR

Media	Cells	Media	Cells
-	+	-	+
-	+	-	+
-	+	-	+
A	D	A	D
A	D	A	D
A	D	A	D
A	D	A	D





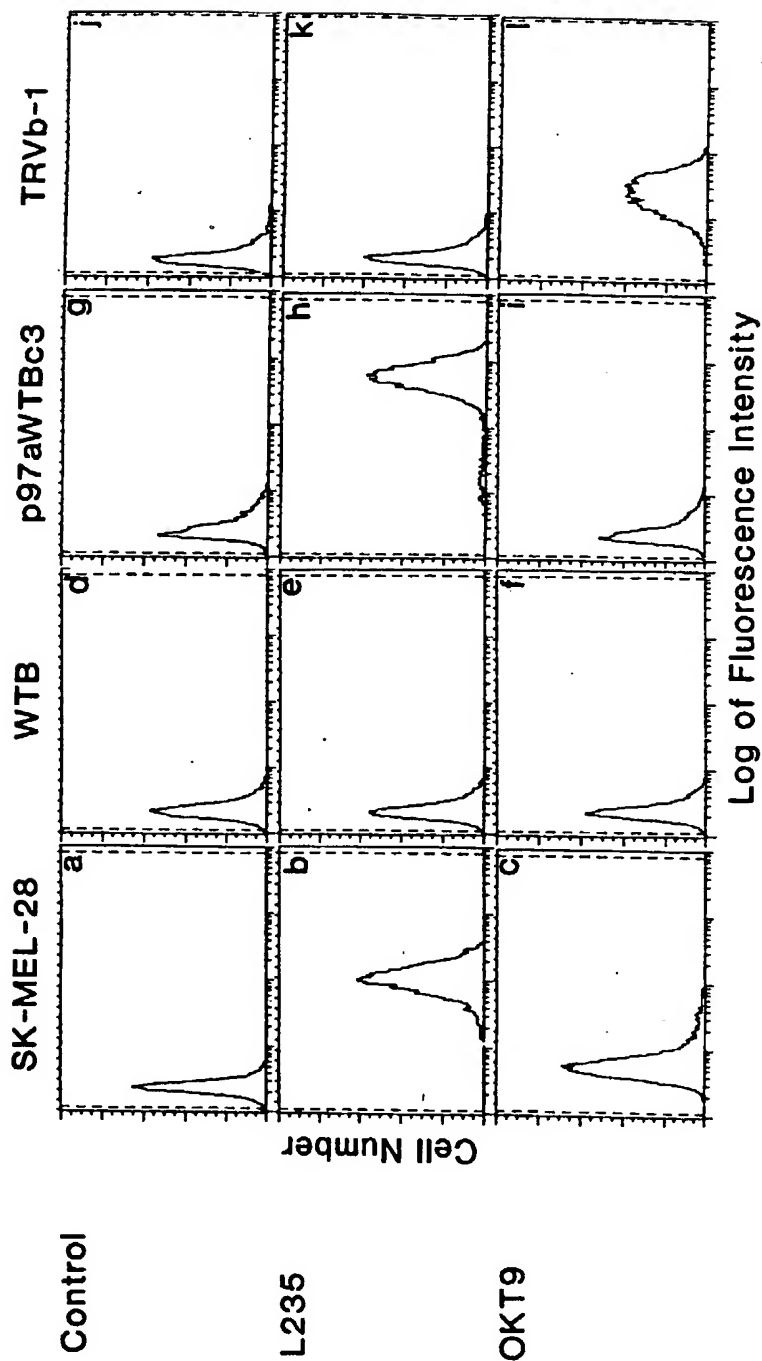
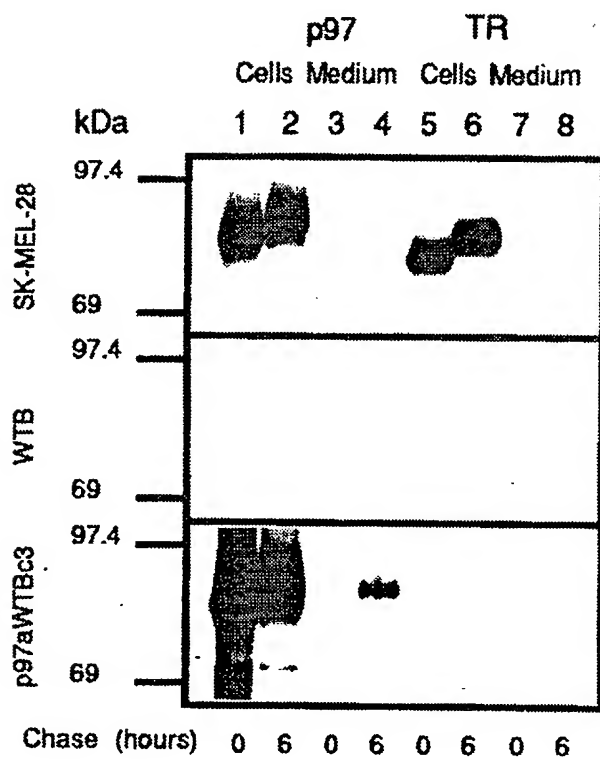


FIGURE 10

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## FIGURE 11



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FIGURE 12

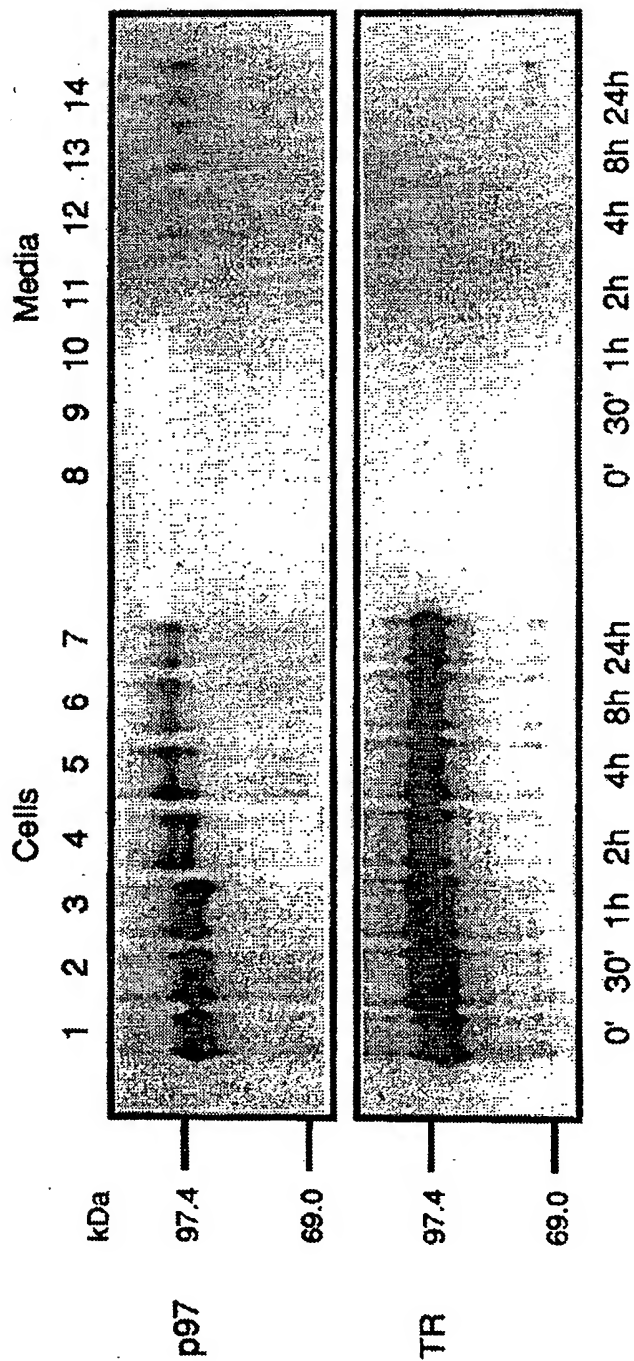
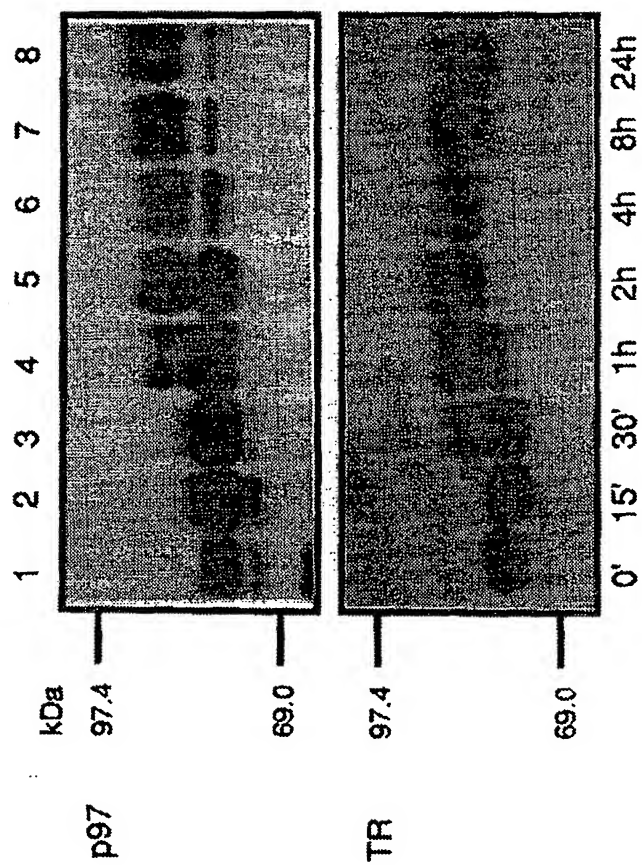


FIGURE 13



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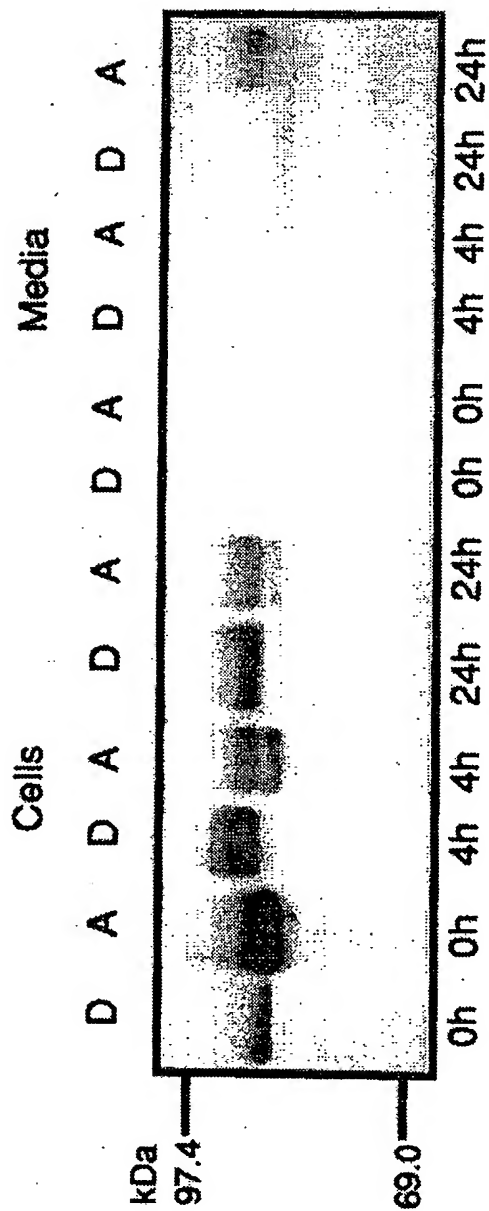


FIGURE 14

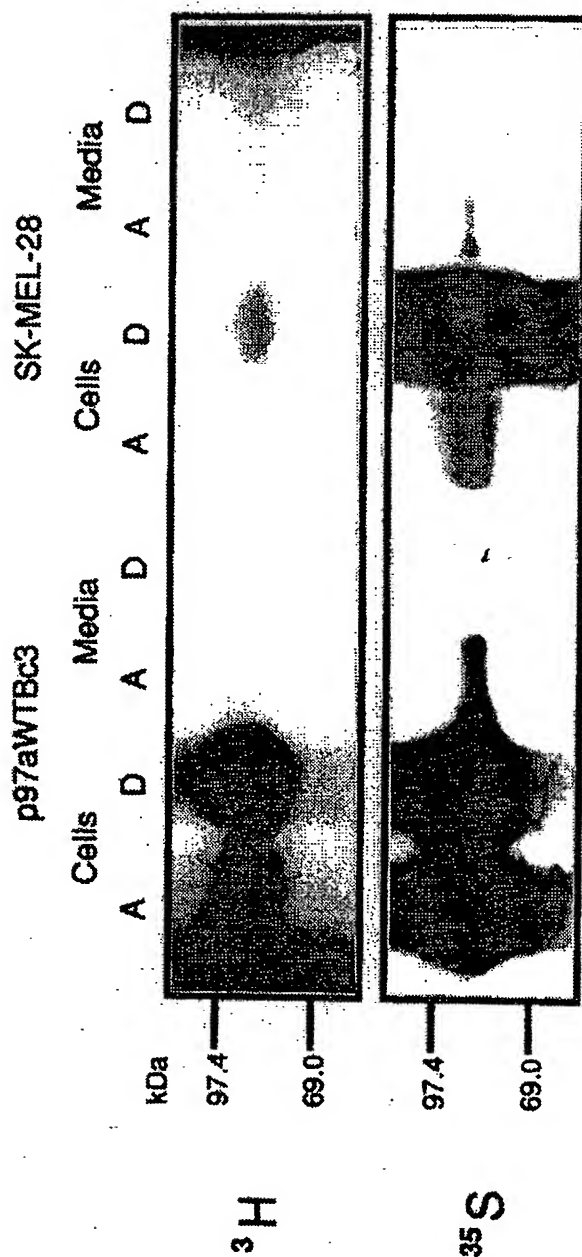


FIGURE 15

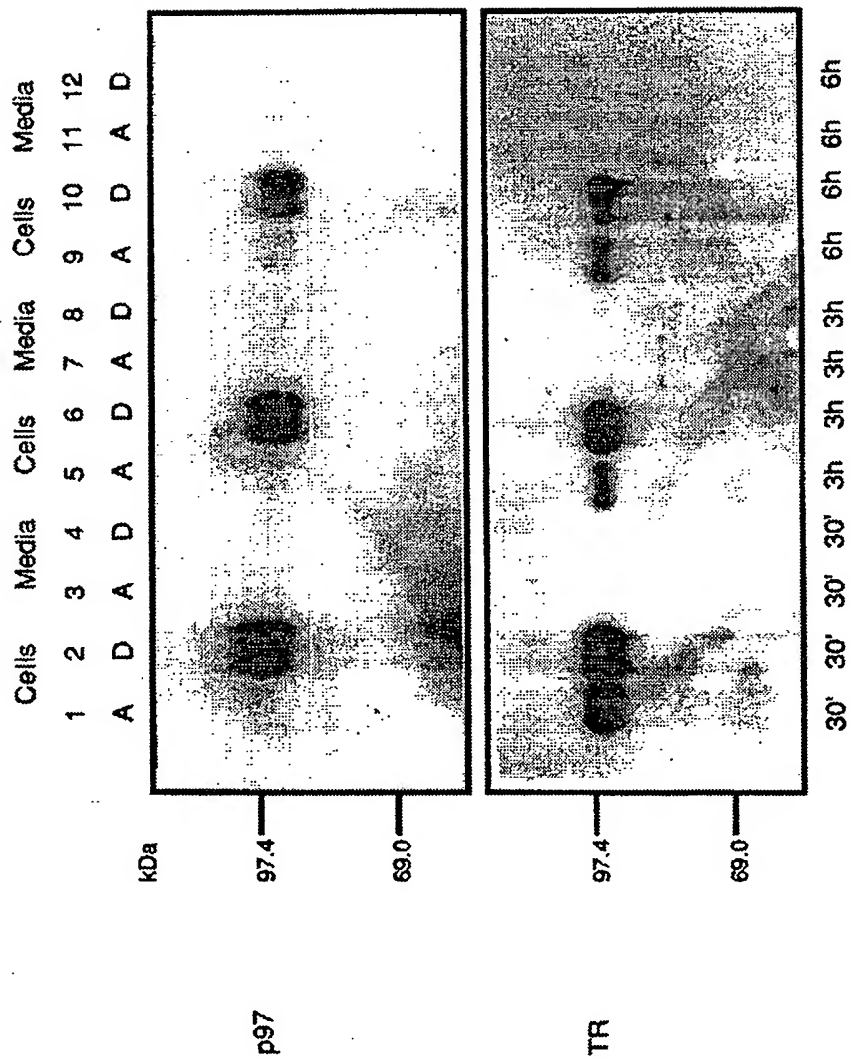
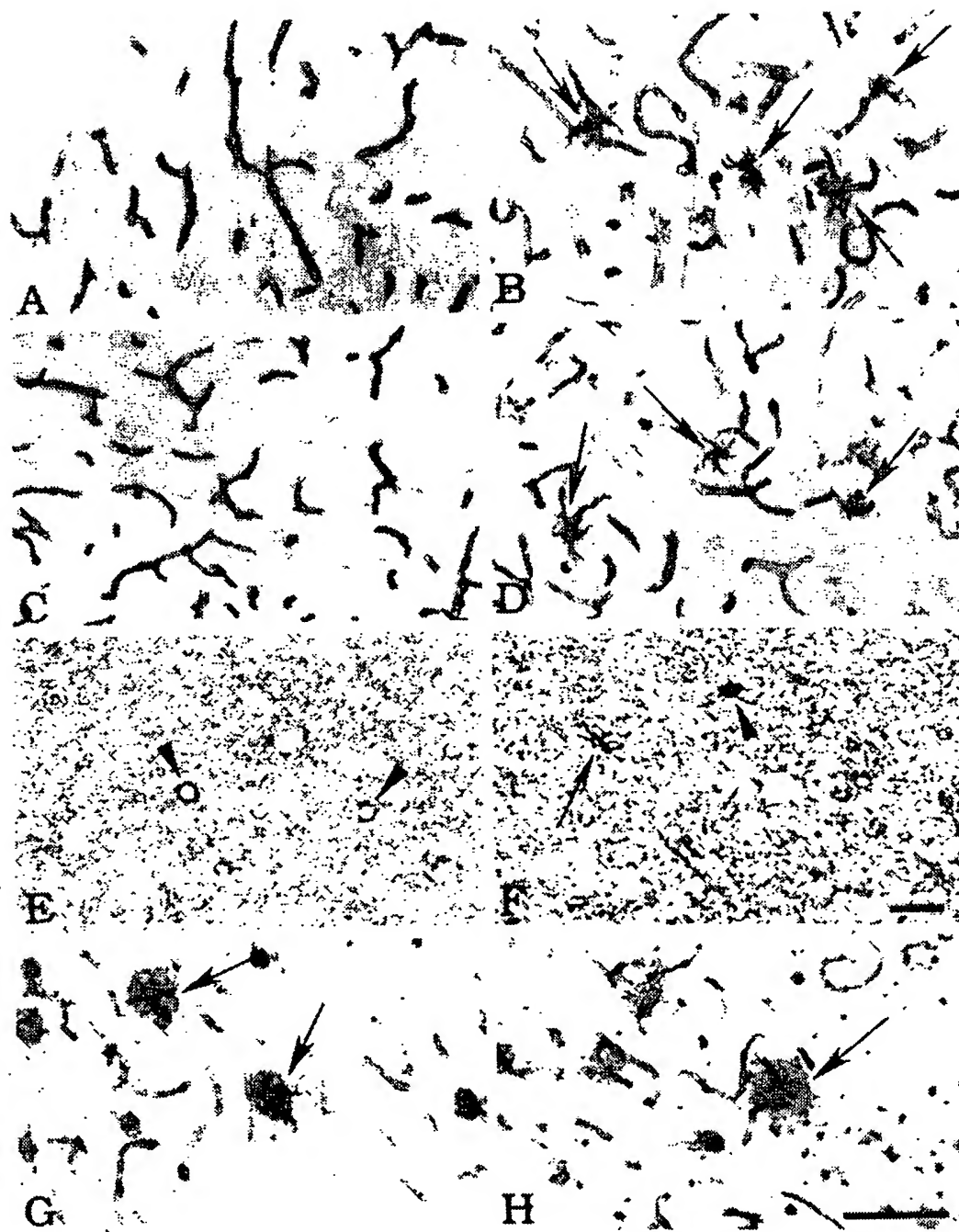


FIGURE 16

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FIGURE 17



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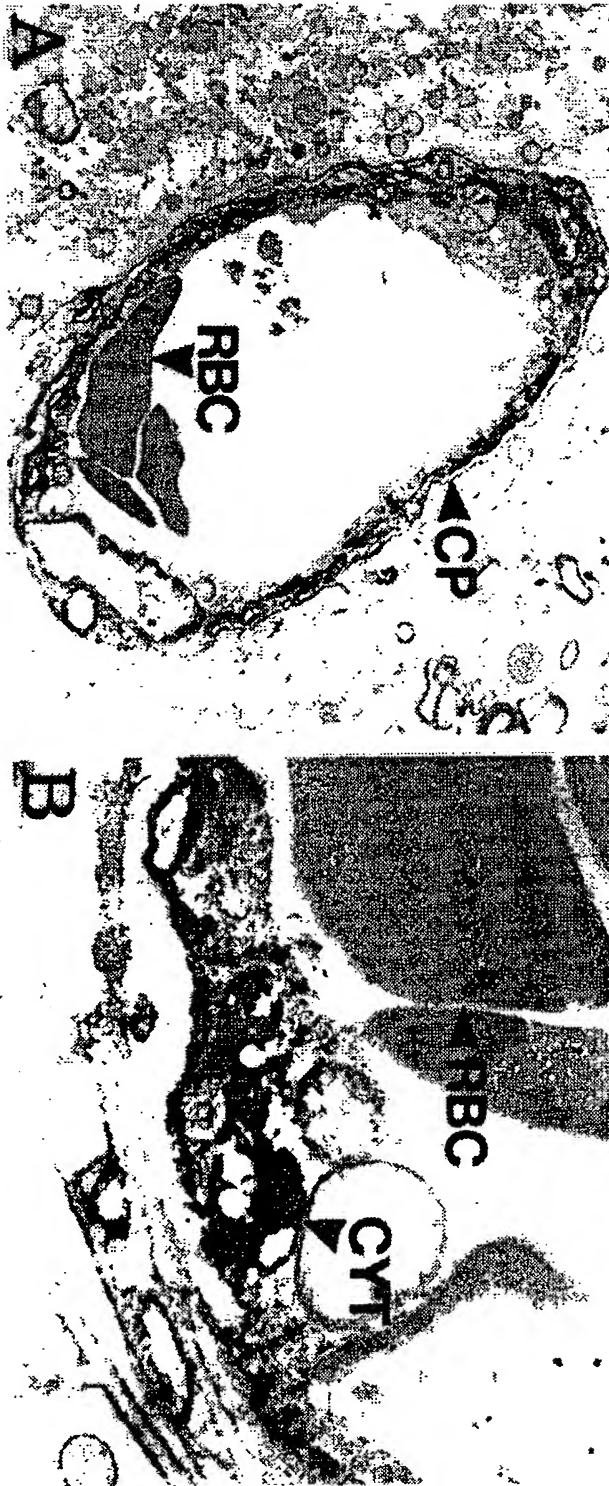


FIGURE 18

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FIGURE 19A

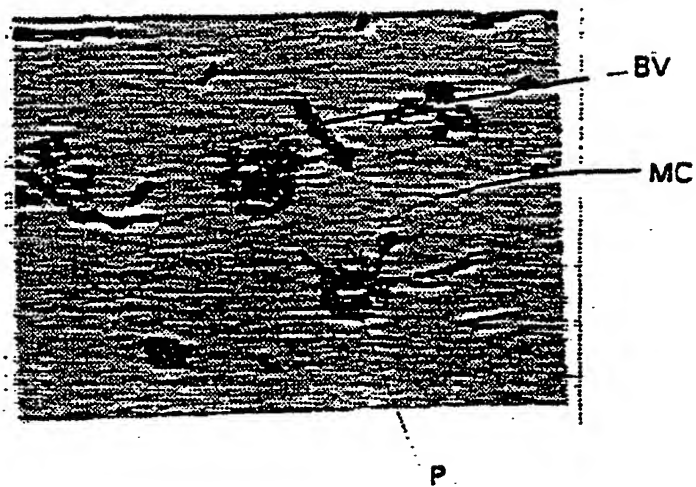
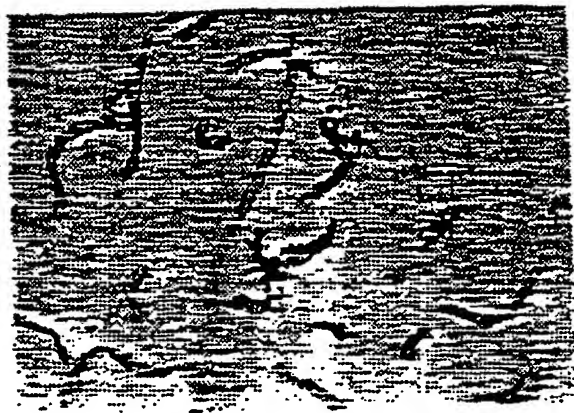


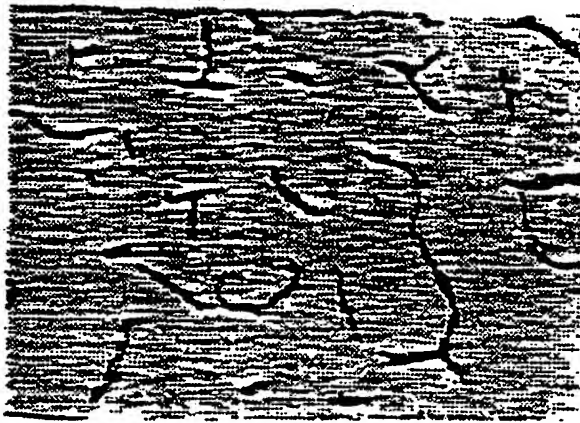
FIGURE 19B



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FIGURE 19C



**SUBSTITUTE SHEET**

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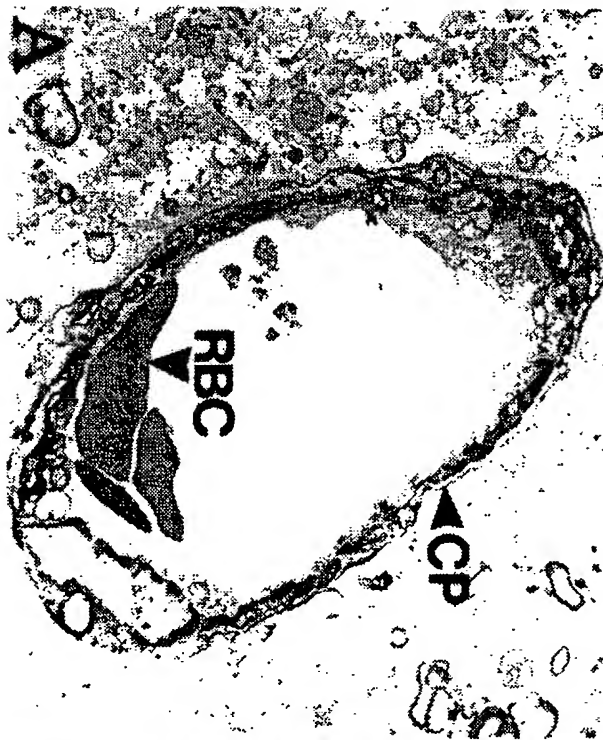


FIGURE 19D

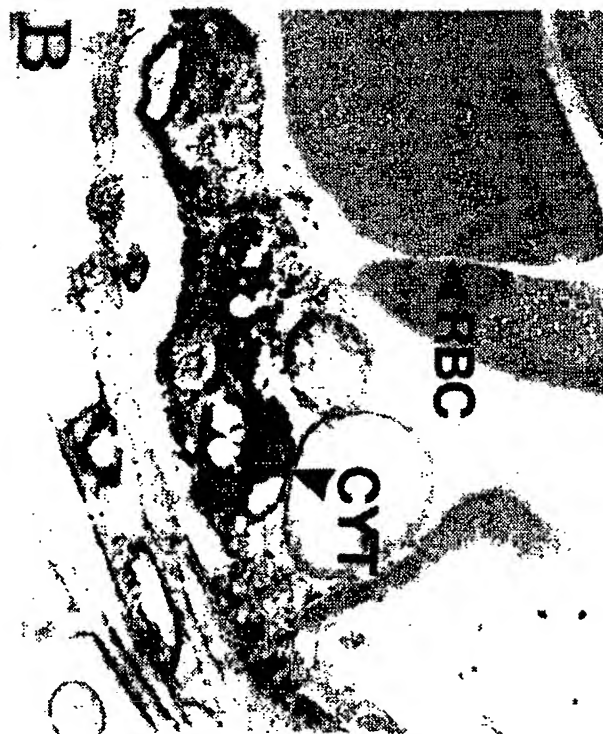


FIGURE 19E

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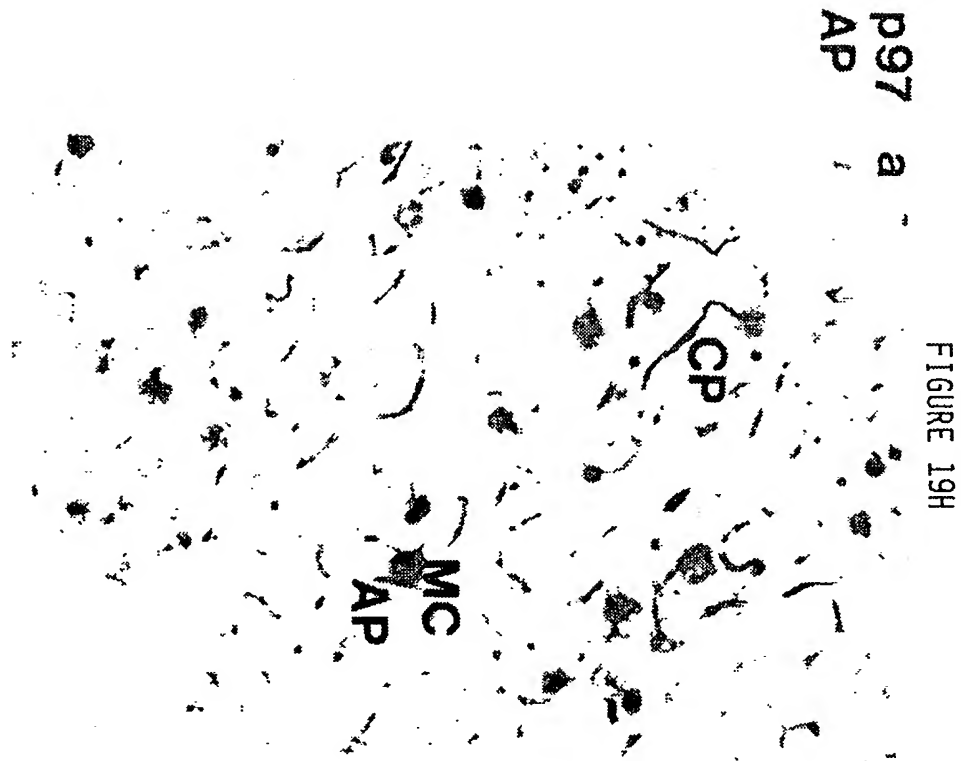


FIGURE 19F

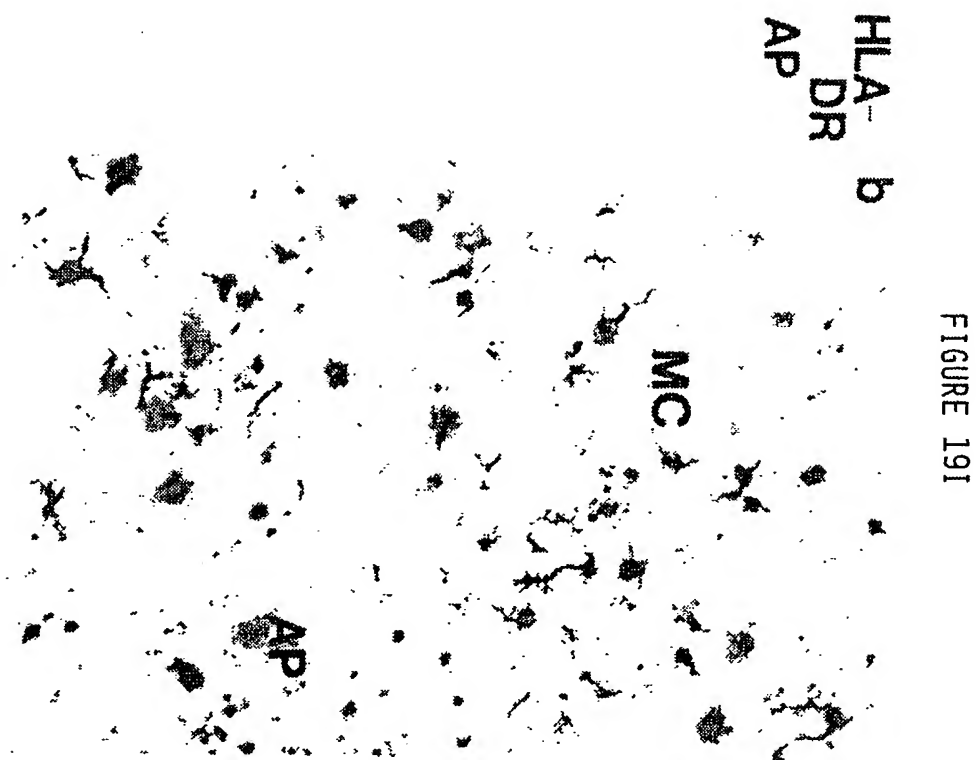
p97



FIGURE 19G



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FIGURE 19J

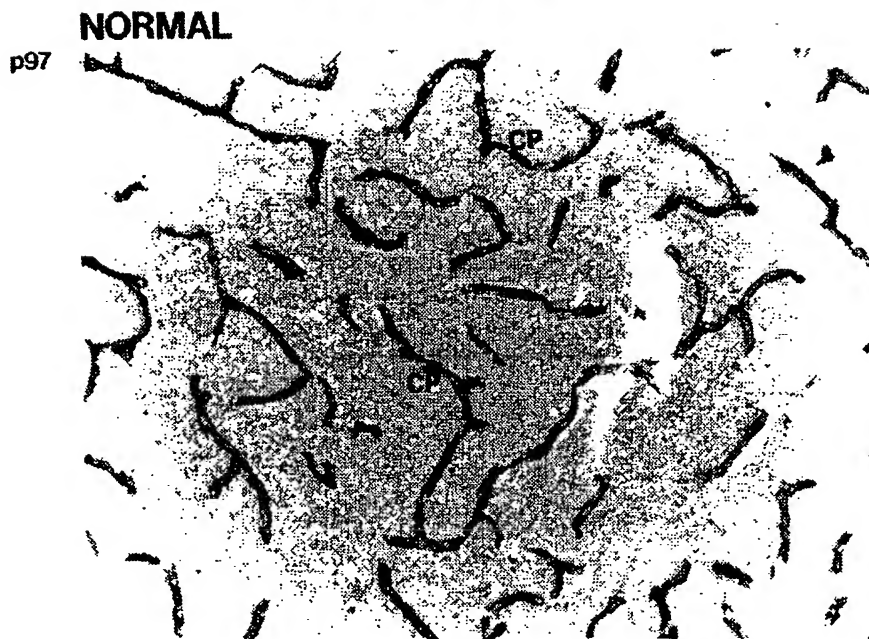


FIGURE 19K



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No PI-PLC

a

Absorbed

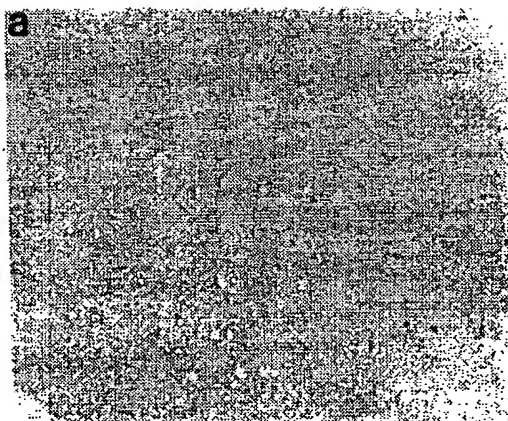


FIGURE 19N

No PI-PLC

b

p97

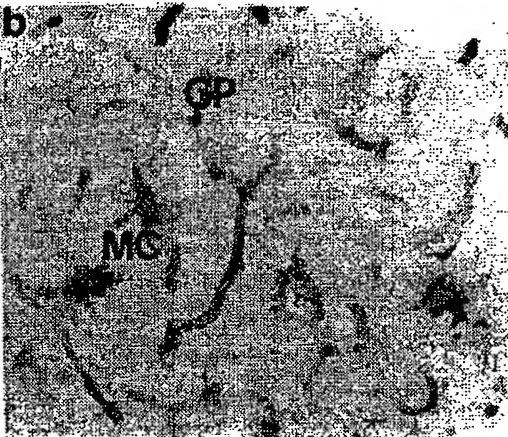


FIGURE 19L

PI-PLC

c

p97

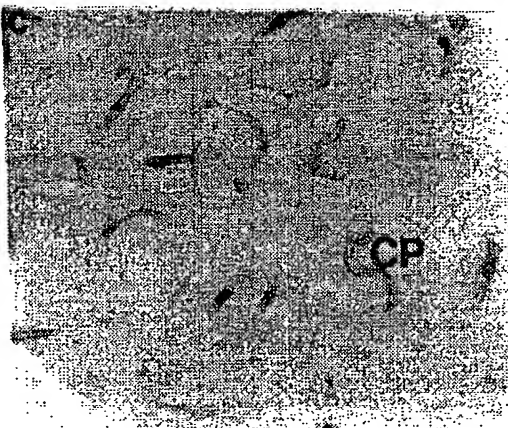


FIGURE 19M

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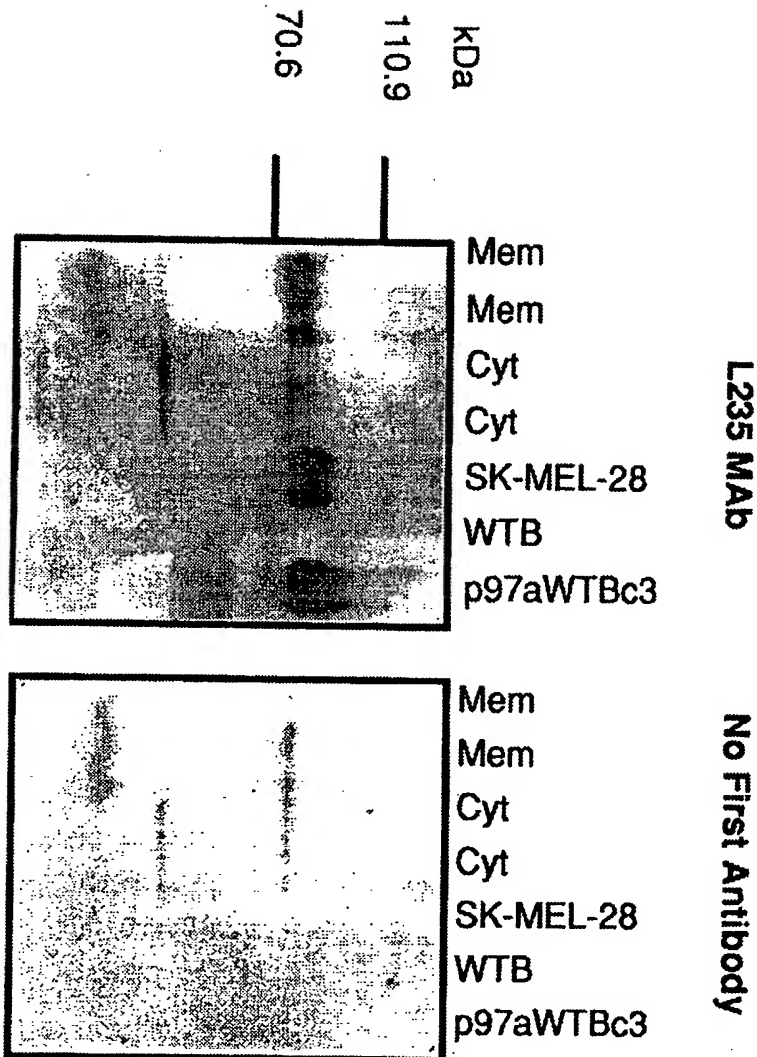


FIGURE 20

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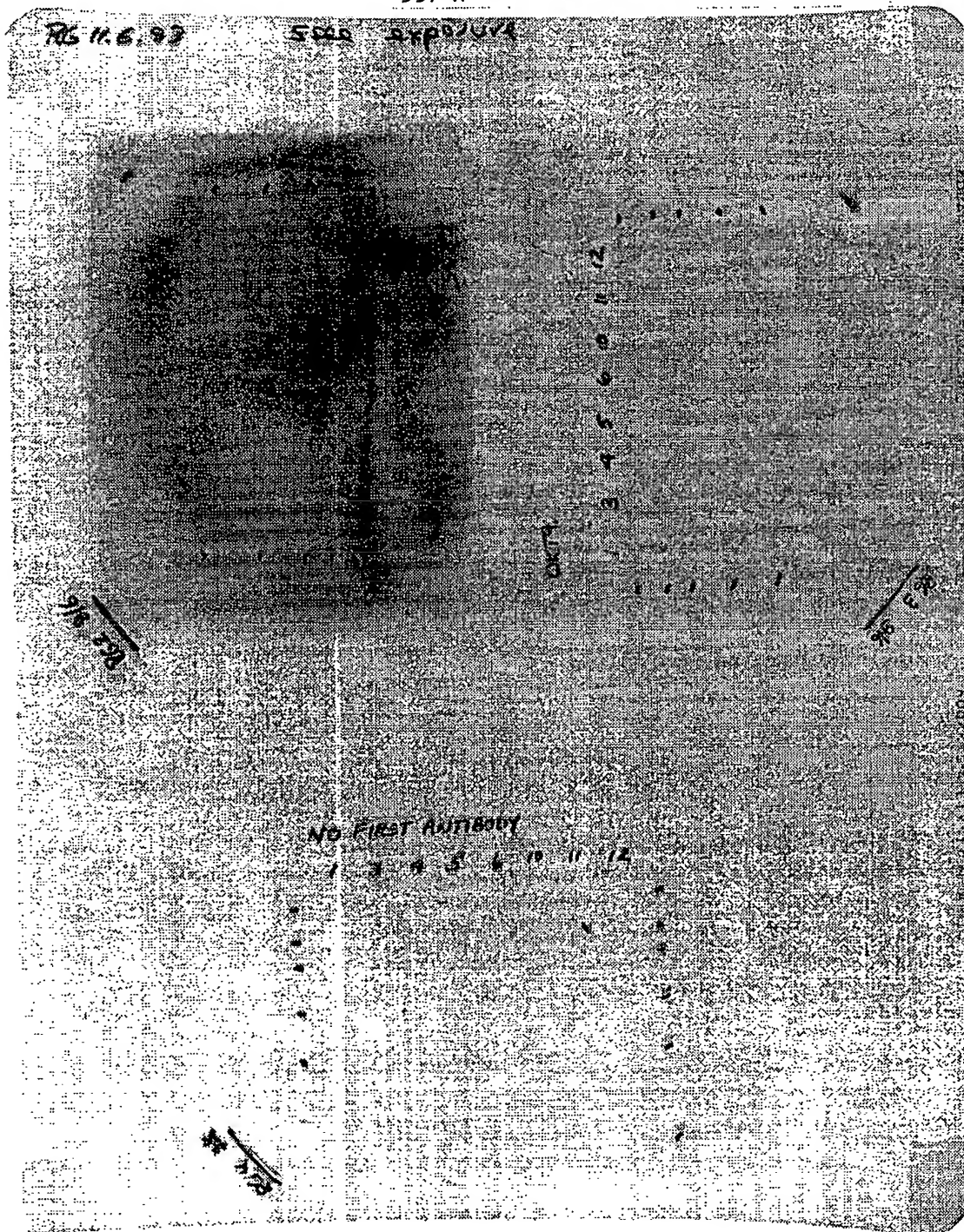


FIGURE 21

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FIGURE 22A

kDa

## Biosynthetic Labelling

Cells

Media

1 2 3 4 5 6 7 8

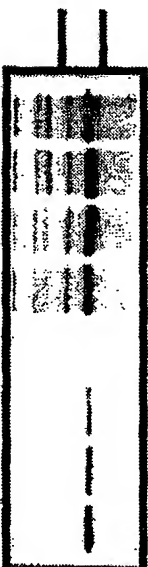
## Cell Surface Labelling

Cells

Media

9 10 11 12 13 14 15 16

p97

97.4  
69.0

TR

97.4  
69.0

FIGURE 22B

0h 4h 8h 24h 0h 4h 8h 24h

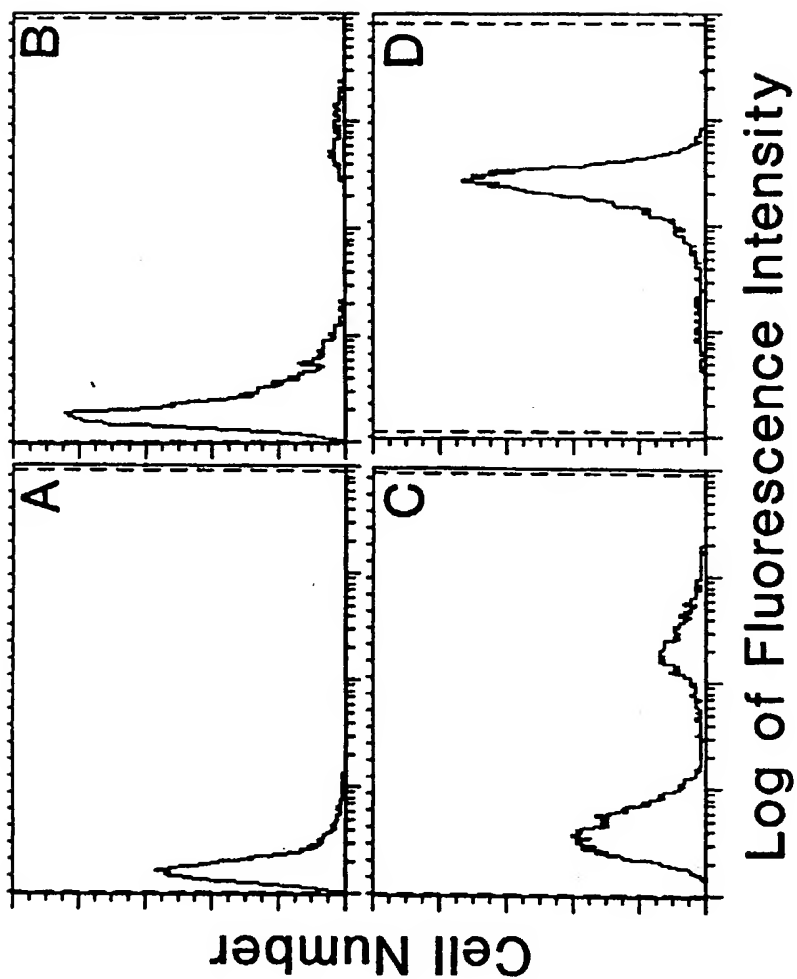
FIGURE 22C



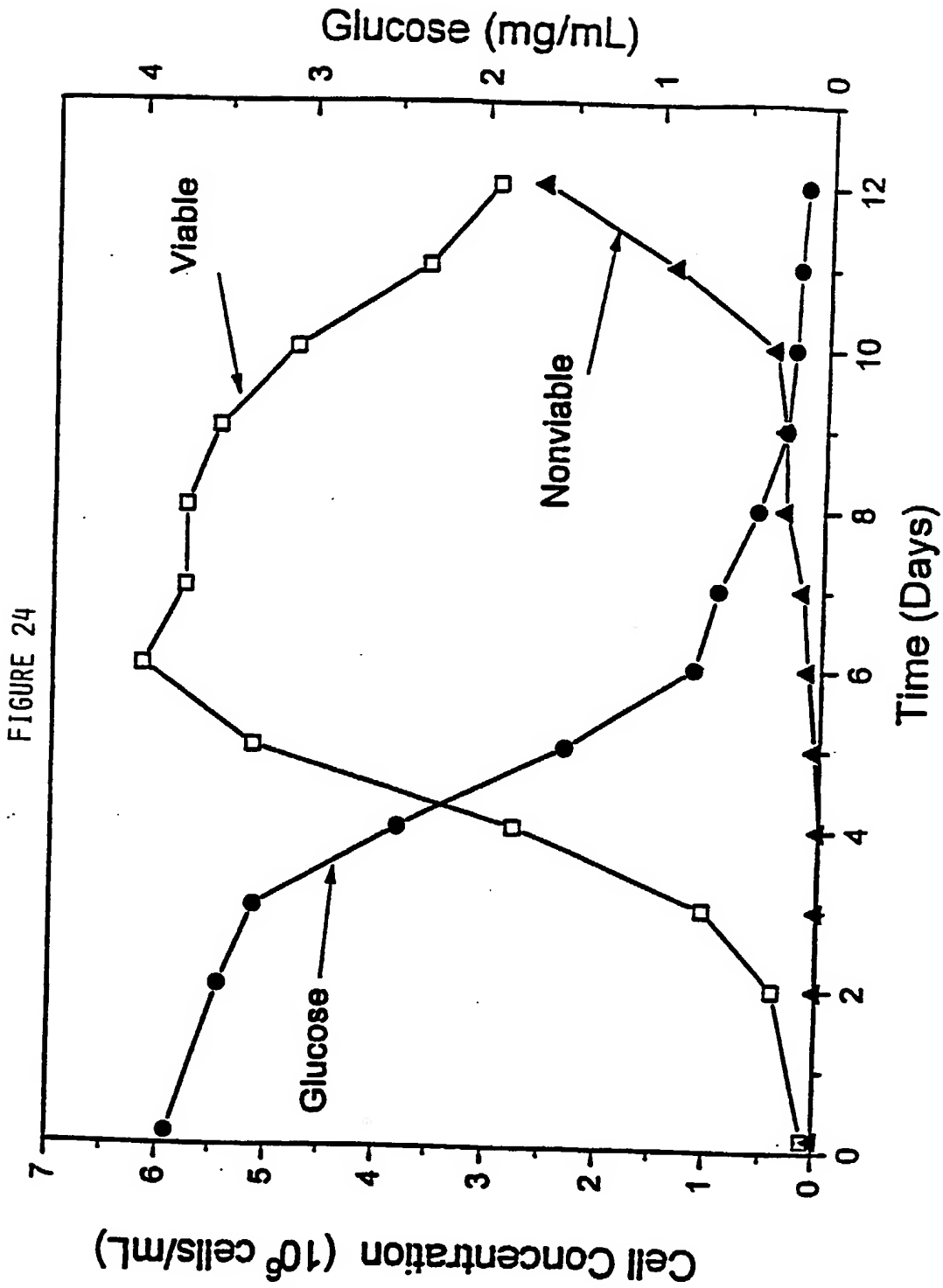
FIGURE 22D

0h 4h 8h 24h 0h 4h 8h 24h

FIGURE 23



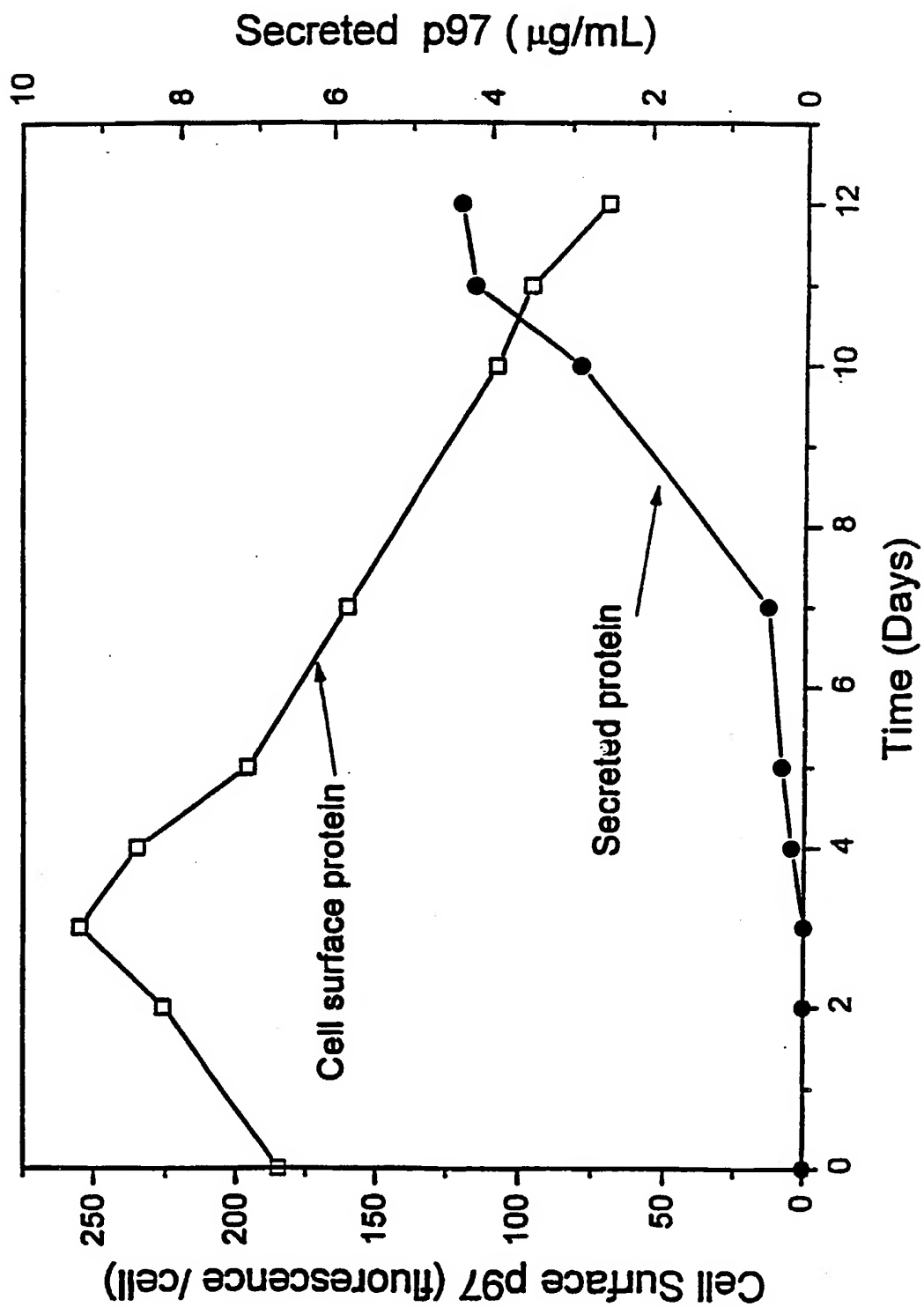
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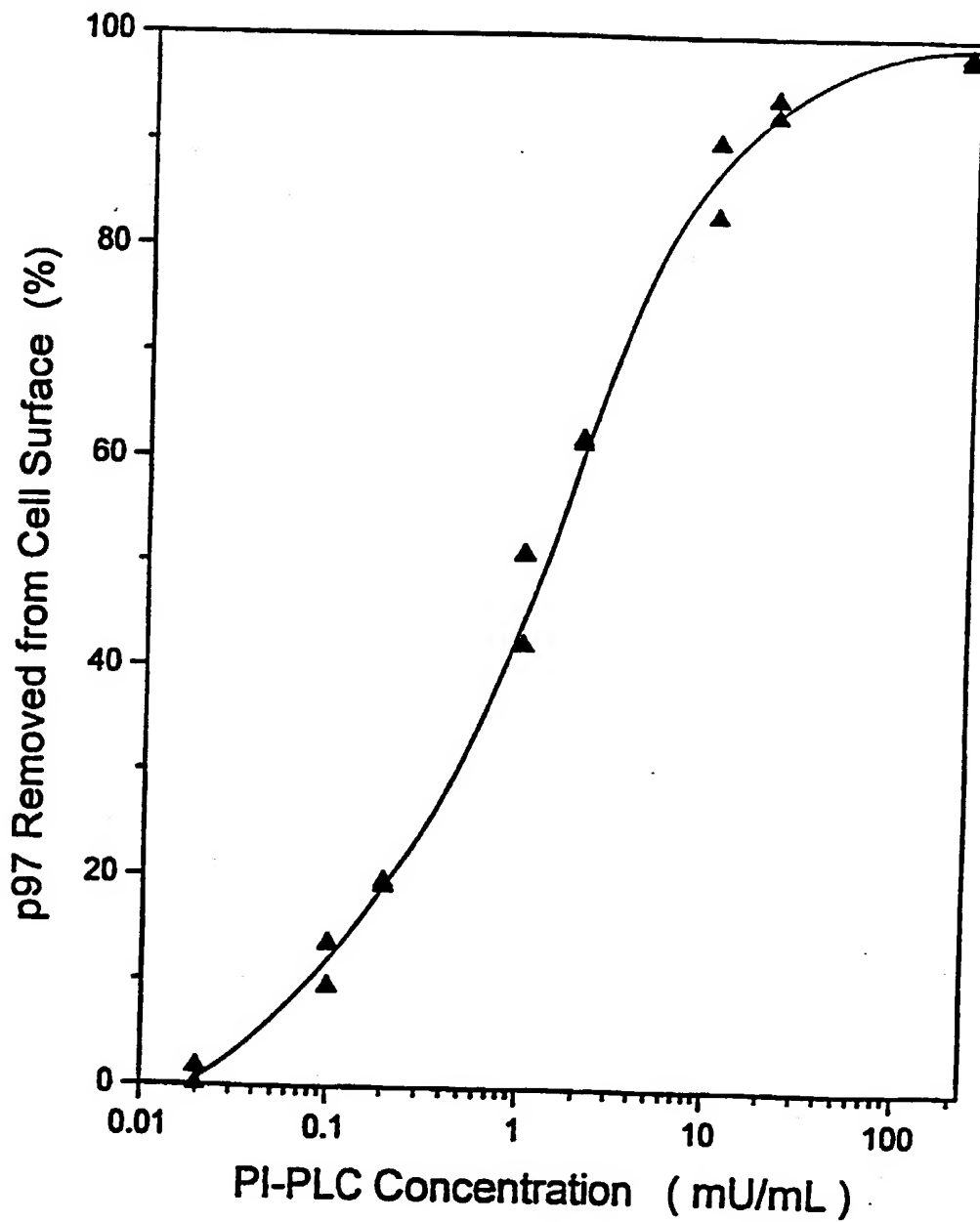
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FIGURE 25



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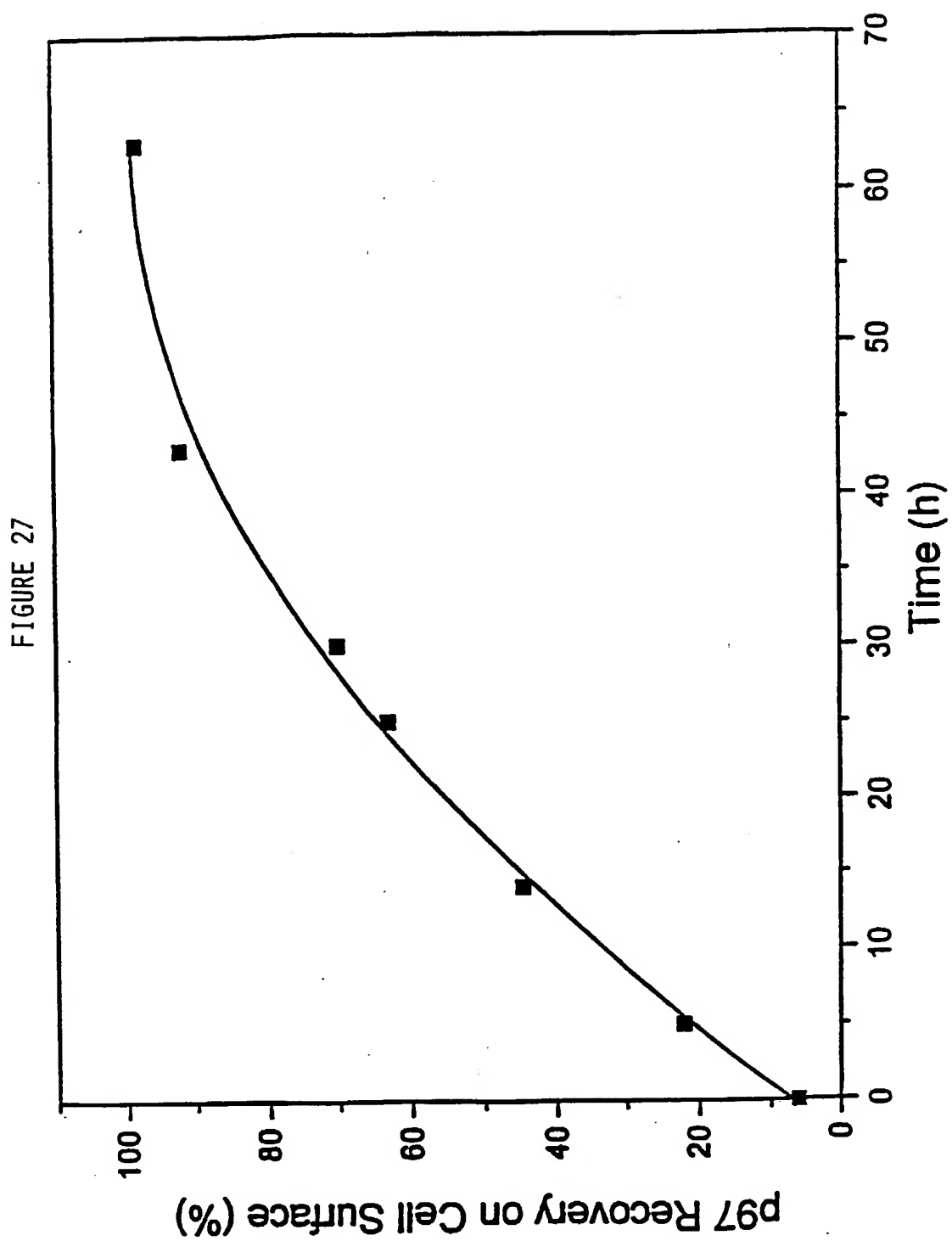
FIGURE 26



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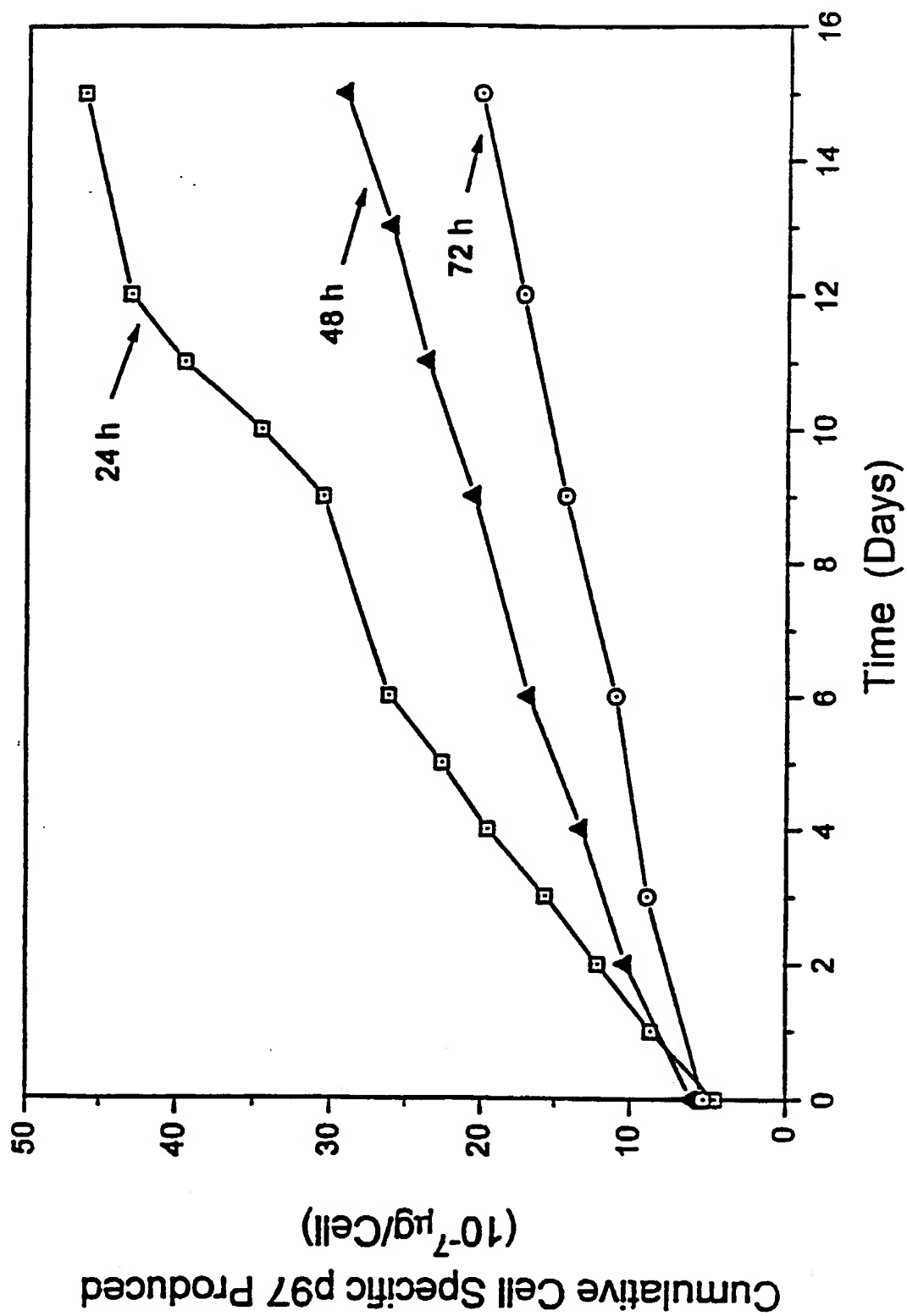
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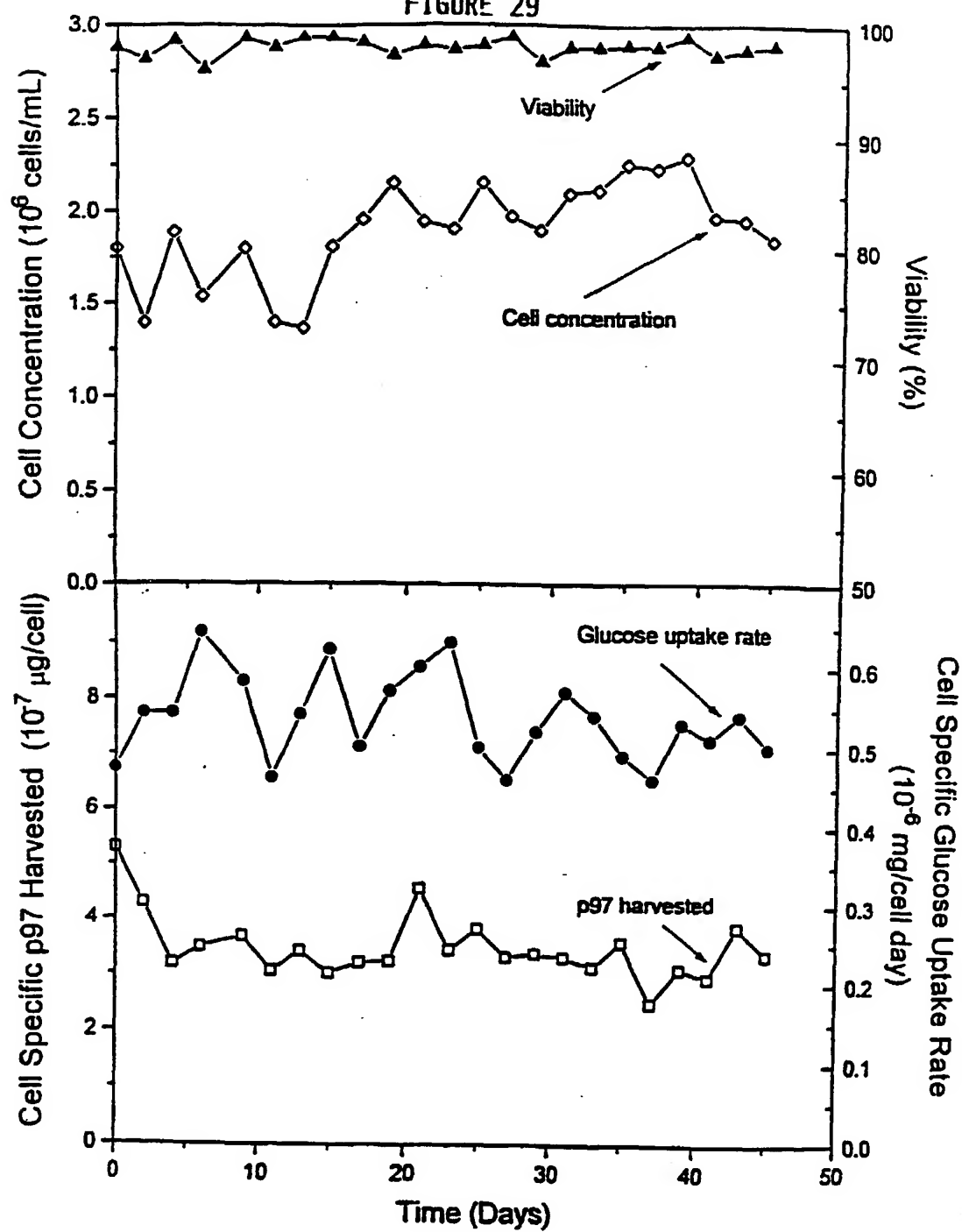
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FIGURE 28



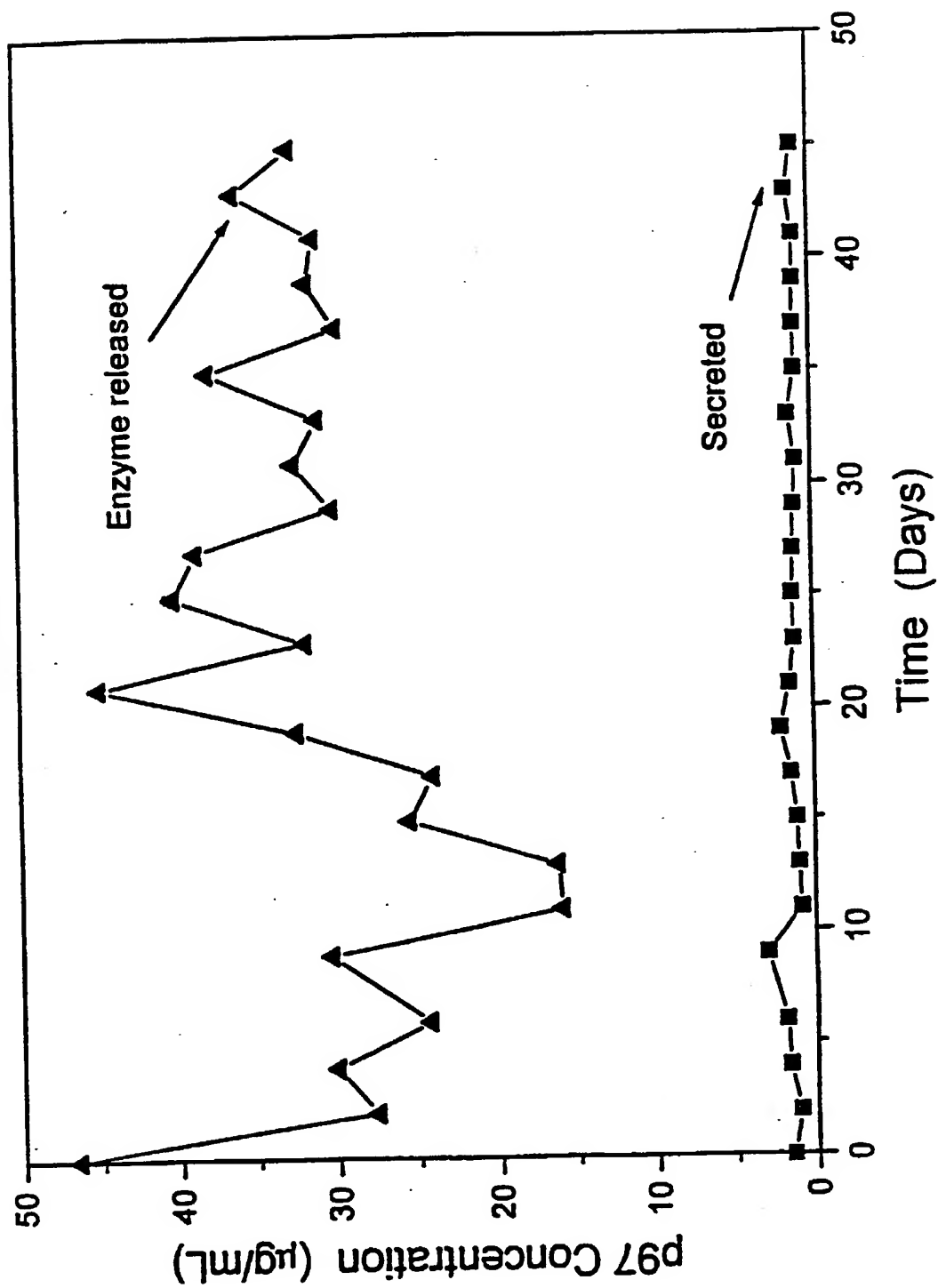
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FIGURE 29



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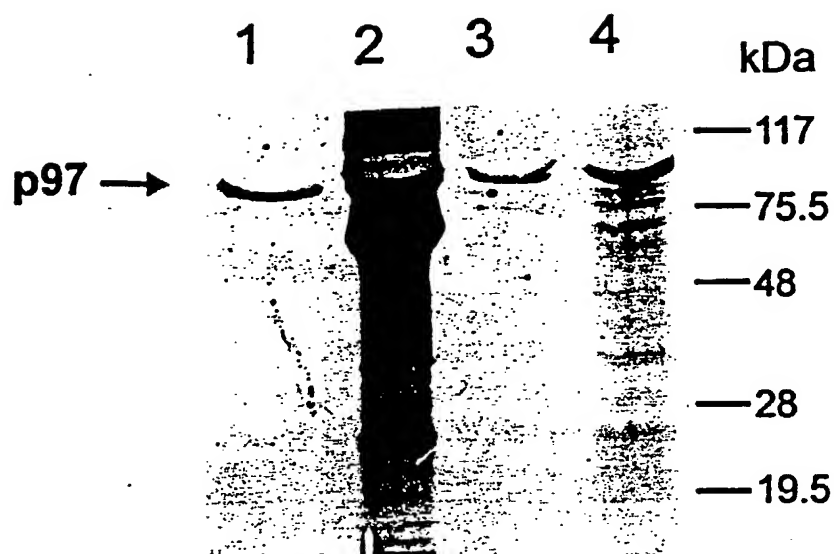
FIGURE 30



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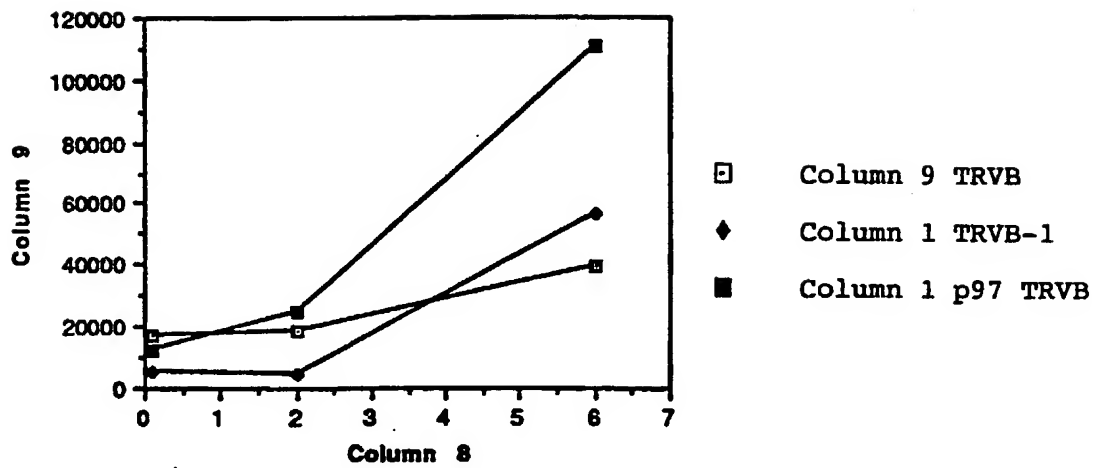
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FIGURE 31



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FIGURE 32



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FIGURE 33A

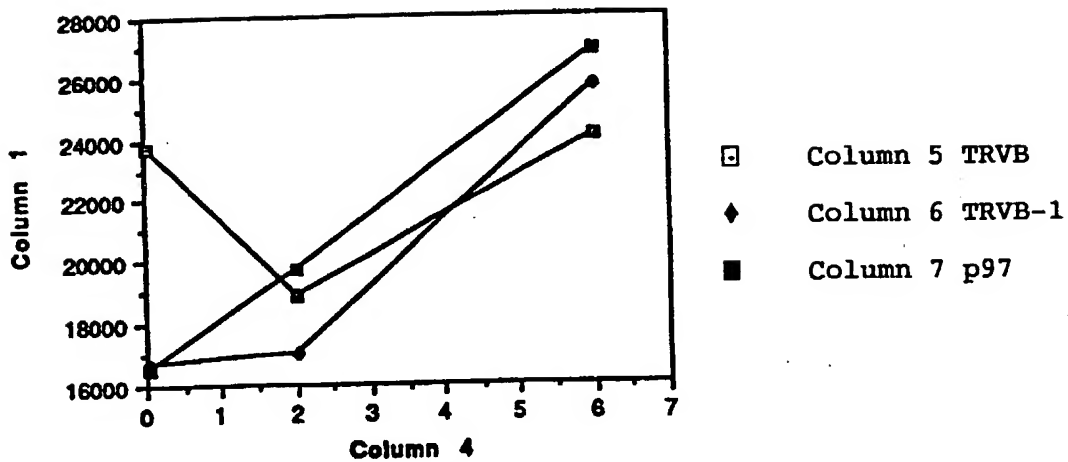
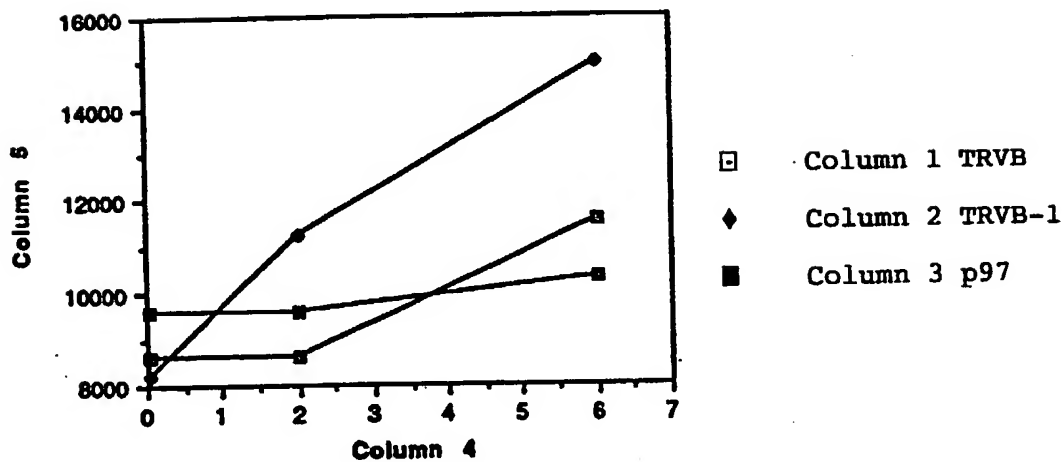


FIGURE 33B



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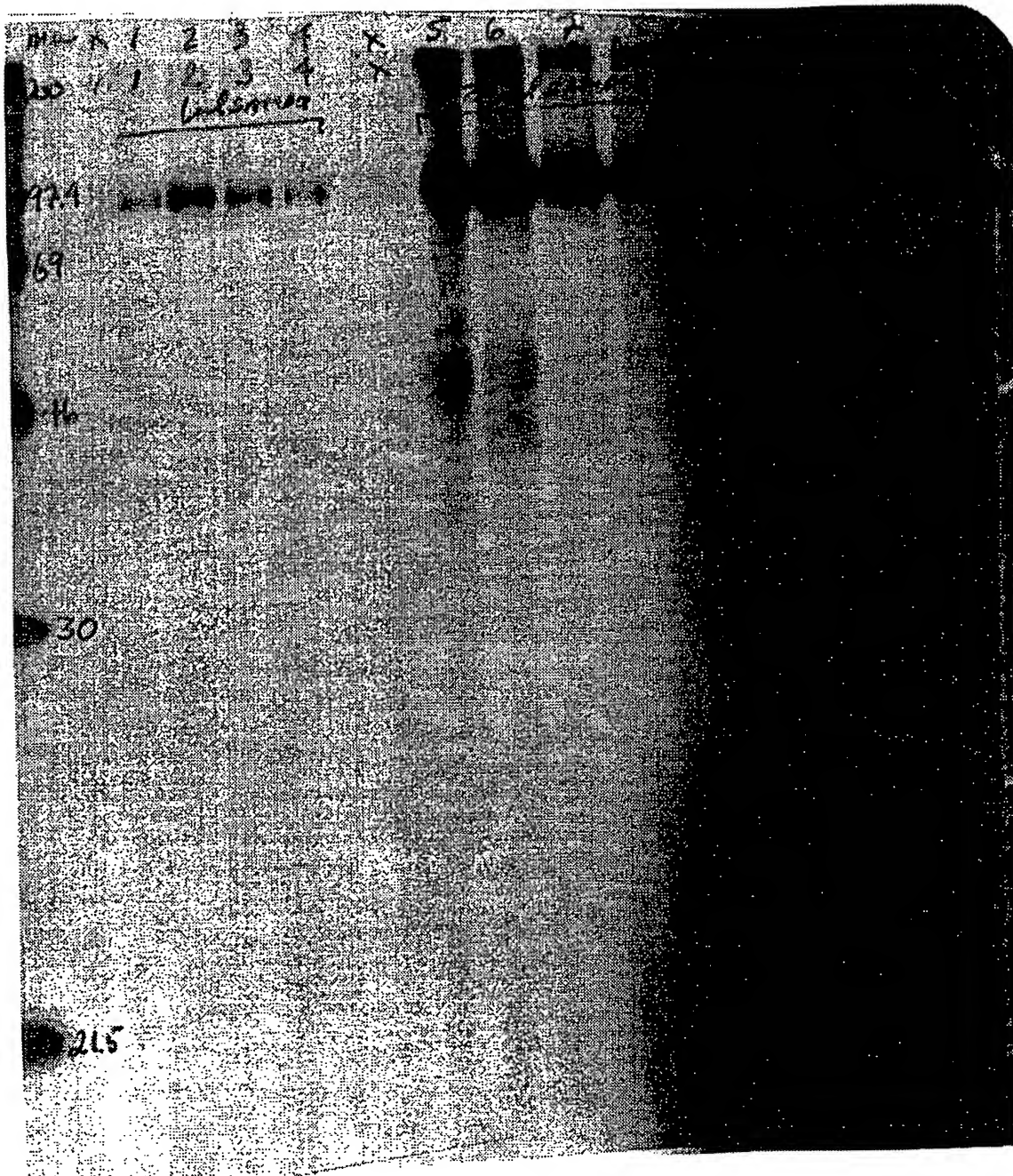


FIGURE 34



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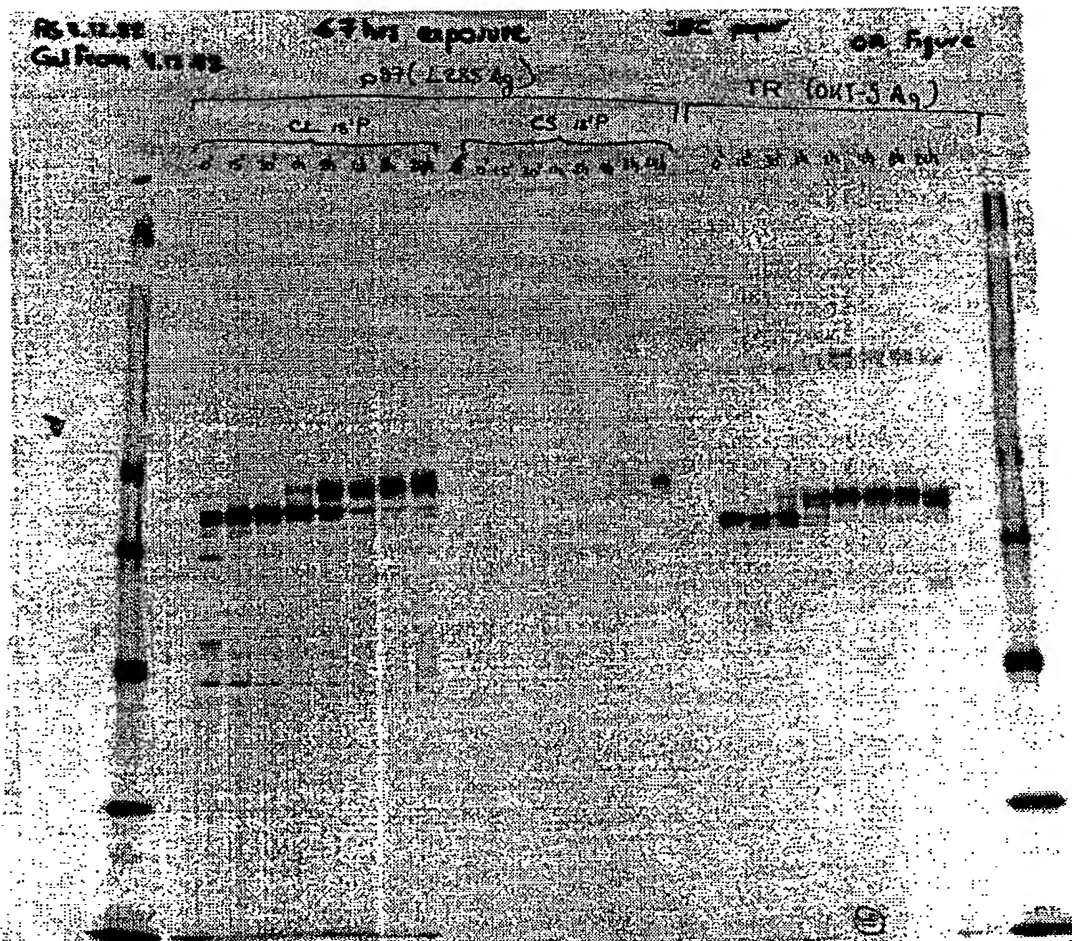


FIGURE 35

## INTERNATIONAL SEARCH REPORT

Inter national Application No  
PCT/CA 93/00272

## A. CLASSIFICATION OF SUBJECT MATTER

IPC 5 C07K13/00 A61K37/02 G01N33/68 C12N5/08

According to International Patent Classification (IPC) or to both national classification and IPC

## B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 5 C07K A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

## C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JOURNAL OF IMMUNOLOGY. vol. 127, no. 2, 1981, BALTIMORE US pages 539 - 546 J. BROWN ET AL 'Structural characterization of human melanoma-associated antigen p97 with monoclonal antibodies' cited in the application see figure 2 ---	1
X	GB,A,2 188 637 (ONCOGEN) 7 October 1987	3-7
Y	see examples 2.2,7 ---	8,9
Y	US,A,4 522 750 (E. ADES ET AL) 11 June 1985 see the whole document ---	8,9
	--- -/--	

☒ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

## \* Special categories of cited documents:

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- "O" document referring to an oral disclosure, use, exhibition or other means
- "P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search

29 November 1993

Date of mailing of the international search report

21-12-1993

Name and mailing address of the ISA

European Patent Office, P.B. 5818 Patentlaan 2  
NL - 2280 HV Rijswijk  
Tel. (+31-70) 340-2040, Tx. 31 651 epo nl,  
Fax (+31-70) 340-3016

Authorized officer

Van der Schaal, C

# INTERNATIONAL SEARCH REPORT

Inter val Application No  
PCT/CA 93/00272

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT		
Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO,A,85 00812 (C. EYLES) 28 February 1985 see the whole document ----	8,9
A	SCIENCE. vol. 249 , 10 August 1990 , LANCASTER, PA US pages 677 - 679 A. LIN ET AL 'Expression of T cell antigen receptor heterodimers in a lipid-linked form' see the whole document -----	2

## INTERNATIONAL SEARCH REPORT

International application No.

PCT/CA93/00272

**Box I** Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:  
because they relate to subject matter not required to be searched by this Authority, namely:  
Remark: Although claims 5,6,9,13 and 14 are directed to methods of treatment of the human body the search has been carried out and based on the alleged effects of the compositions.
2. ☐ Claims Nos.:  
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:  
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

**Box II** Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.